THE ROLE OF CALCIUM IN PANCREATIC ENZYME SECRETION:

USE OF THE DIVALENT CATION IONOPHORE A23187 AND THE ANTIBIOTIC CHLOROTETRACYCLINE AS FLUORESCENCE PROBES TO STUDY INTRACELLULAR CALCIUM REDISTRIBUTION DURING STIMULUS-SECRETION COUPLING

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ABSTRACT

The divalent cation ionophore, A23187, and the antibiotic chlorotetracycline (CTC) were used as fluorescence probes to study the role of calcium in stimulus-secretion coupling in dissociated pancreatic exocrine cells. A23187 was used to promote calcium entry into the cytosol from either the extracellular space or from intracellular stores and thereby artificially trigger secretion. CTC was used as a non-perturbing probe of divalent cations associated with membranes in acinar cells and of changes in ion distribution occuring during stimulation with physiological secretagogues.

Intracellular uptake of A23187 and the increased release of amylase and lactate dehydrogenase (LDH) accompanying ionophore uptake was studied using dissociated acinar cells prepared from mouse pancreas. Easily detected changes in the fluorescence excitation spectrum of A23187 upon transfer of the ionophore from a Tris-buffered Ringar's to cell membranes were used to monitor A23187 uptake. Uptake was rapid in the absence of extracellular Ca^{++} and Mg^{++} ($t_{\frac{1}{2}}$ = 1 min) and much slower in the presence of Ca^{++} or Mg^{++} ($t_{\frac{1}{2}}$ = 20 min). Cell-associated ionophore was largely intracellular as indicated by fluorescence microscopy, lack of spectral sensitivity to changes in extracellular Ca^{++} and Mg^{++}, and by equivalent interaction of ionophore with membranes of whole and sonicated cells.

A23187 (10 uM) increased amylase release 200% in the presence of extracellular Ca^{++} and Mg^{++}. In the absence of Ca^{++} (but in the presence of Mg^{++}) A23187 did not increase amylase release. A23187 (10 uM) also produced Ca^{++}-dependent cell damage, as judged by increased LDH release, increased permeability to trypan blue, and by disruption of cell morphology. The cell damaging and amylase releasing properties of A23187 were distinguished by their time course and dose-response relationship. A23187 (1 uM) increased amylase release 140% without increasing LDH release or permeability to trypan blue.

Studies with chlorotetracycline (CTC) were carried out using dissociated pancreatic acini. Acini which were preincubated with CTC, 100 uM, and resuspended in medium containing no CTC, displayed a slow loss of fluorescence. Addition of bethanechol or caerulein to these CTC-loaded acini resulted in a rapid loss of fluorescence intensity in the first 5 minutes of secretagogue action and a three fold increase in chemical outflux of CTC. The fluorescence loss elicited by bethanechol was blocked by atropine while that elicited by caerulein was not; fluorescence responses to either secretagogue were observed in the presence or absence of extracellular calcium. The fluorescence decrease elicited by bethanechol was dose dependent with half-maximal and maximal responses occuring at 20 uM and 100 uM respectively. Fluorescence reponses were elicited by all concentrations of bethanechol that stimulated amylase release but maximal secretion was achieved at a lower concentration, 30 uM, than that required for maximal fluorescence response.

Fluorescence spectra of unstimulated, CTC-loaded acini were like those of Ca^{++}-complexed CTC while spectra of bethanechol- or caerulein-stimulated acini were like that of Mg^{++}-complexed CTC. It is concluded that stimulation of acinar cells is accompanied by a release of intracellular calcium that is reflected in a preferential dissociation of Ca^{++}-CTC complexes and partitioning of the free CTC out of the membrane. In contrast, acini loaded with 1-anilino-8-naphthalenesulfonate (ANS) or oxytetracycline, fluorescence probes that are relatively less sensitive to divalent cations, showed no fluorescence changes upon secretory stimulation.

Application of the mitochondrial uncoupler ClCCP or the oxidative phosphorylation inhibitors antimycin A or NaCN rapidly decreased the fluorescence of CTC-loaded acini before but not after stimulation with bethanechol. This suggested that these agents acted to decrease CTC fluorescence at the same site as did bethanechol, possibly at mitochondria or a site requiring ATP for calcium sequestration.

Fluorescence microscopy of isolated pancreatic acinar cells during CTC efflux showed two patterns of emission that were superimposed. A uniform fluorescence eminated from areas known to contain endoplasmic reticulum while an intense punctate fluorescence was observed around the nucleus and zymogen granule cluster. Similar point-like emission was observed in isolated liver cells and from the elipsoidal region of retinal photoreceptors - areas known to be packed with mitochondria. Therefore, the punctate pattern could be from mitochondria or another calcium sequestering organelle in the pancreatic acinar cell and this may be the site from which calcium is released during secretory stimulation.

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TO

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I. INTRODUCTION

Pancreatic acinar cells synthesize, process, and then store digestive enzymes in membrane bound granules in a series of steps carefully documented by Palade and coworkers (Caro & Palade, 1964; Jamieson & Palade, 1967a,b; Jamieson, 1972; Palade, 1975). Release of these stored secretory proteins is a temporally separate event initiated by interaction of a secretagogue with a plasma membrane receptor. Receptor activation leads to intracellular events termed "stimulus-secretion coupling" (Douglas & Rubin, 1961; Douglas, 1968) which in turn trigger exocytosis of secretory granules. In the pancreas, as in many secretory tissues, exocytosis is thought to be triggered by a rise in cytoplasmic calcium activity. How the pancreatic secretagogues acetylcholine and cholecystokinin-pancreozymin might bring this about is the main concern of this thesis.

A. Stimulus-Secretion Coupling: The Concept

1. An Analogy

The role of calcium as a regulator of intracellular processes was first demonstrated (Heilbrun & Wiercinski, 1947) and is clearly documented in muscle (Sandow, 1970; Fuchs, 1974). The cytoplasm of these cells is considered to have a very low calcium activity (less than 10^{-6} M) when the cell is at rest (Portzehl, Caldwell & Ruegg, 1964; Ashley, 1970). Muscle contraction is initiated by a rapid rise in the level of ionized calcium (Ashley & Ridgeway, 1970; Blinks, 1973; Taylor, Rudel & Blinks, 1975). This calcium then binds to a subunit of the regulatory protein troponin which, in turn, allows the contractile proteins actin and myosin to assume a force generating relationship (Weber & Murry, 1973; Gergely, 1976). It was this use of calcium as an intermediate signal to transduce membrane depolarization

into muscle contraction that Sandow (1952) termed "excitation-contraction coupling". The source of this "trigger" calcium was found to vary according to the type of muscle. It is released from a specialized calcium accumulating organelle, the sarcoplasmic reticulum, in skeletal muscle (Poldolsky, 1975); it flows from the extracellular space in cardiac muscle (Langer, 1976); and it comes from a combination of these two sources in most smooth muscles (Somlyo, 1972; Bohr, 1973; Somlyo & Somlyo, 1975). In all cases, however, it is an increase in cytosol calcium activity which triggers contraction.

When Douglas found that extracellular calcium was essential for secretion from the adrenal medulla (Douglas & Rubin, 1961) and the posterior pituitary (Douglas & Poisner, 1964a,b), he used the precedent set by muscle to postulate that calcium was acting as an intracellular messenger for release of secretory products -- hence the term stimulus-secretion coupling. Most evidence gathered since then has strengthened this analogy.

2. Intracellular Calcium Homeostasis

Use of calcium sensitive luminescent proteins (e.g. aequorin) and dyes (e.g. murexide, arsenazo III) has established that resting levels of free calcium ions in nerve (DiPolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976), muscle (Ashley, 1970), and secretory cells (Rose & Loewenstein, 1976) is exceedingly low. The best measurements have been made in squid axons where the light output of aequorin (approximately proportional to calcium activity) or the absorbance of arsenazo III can be calibrated by intraaxonal perfusion of Ca^{++}-EGTA buffers having extremely low but defined calcium concentrations. Two independent studies using this approach found intraaxonal calcium activities of less than 100 nM (Baker, Hodgkin & Ridgeway, 1971; DiPolo et al., 1976). Studies using aequorin in large secretory cells such as the presynaptic nerve terminal at the squid

giant synapse (Llinas & Nicholson, 1975) and the blowfly salivary gland (Rose & Loewenstein, 1976) also estimate that the intracellular calcium activity is less than 1 uM.

In contrast to these low intracellular activities, the total calcium contained in most tissues (e.g. as measured by atomic absorption spectrometry) is much higher, usually in the range of 0.3 to 3 mmoles per kg wet wt (Bianchi, 1968; Irving, 1973). This implies that most calcium is tightly bound or sequestered in noncytoplasmic compartments. Indeed, there is already overwhelming evidence for calcium sequestration in two subcellular organelles -- mitochondria and sarcoplasmic reticulum. Mitochondria isolated from both excitable and nonexcitable tissue have been shown to avidly accumulate calcium (Carafoli & Lehninger, 1971) and are able to maintain calcium concentration gradients of over 1000 when exposed to external calcium levels in the micromolar range (Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965; Mela & Chance, 1968; Lehninger, 1970). Using isotopic calcium exchange techniques, Borle has identified a large pool of slowly exchangable calcium in cultured kidney cells (Borle, 1972,1973; Borle & Anderson, 1976). This pool appears to be mitochondrial since it is eliminated in the presence of antimycin A and dicumerol (inhibitors of mitochondrial calcium uptake) and its size increases in the presence of extracellular phosphate, an ion that is permissive for mitochondrial calcium uptake (Lehninger, 1974). Subcellular fractionation techniques, even in studies that have been particularly careful in accounting for artifactual redistribution have indicated that a large part of total cell calcium is found in mitochondrial fractions (Clemente & Meldolesi, 1975; Van Rossum, Smith & Breton, 1976).

In blowfly salivary glands, aequorin has been used to show that calcium injected intracellularly is quickly bound or sequestered and that this "mopping up" is considerably retarded when mitochondrial inhibitors are present (Rose & Loewenstein, 1975, 1976). Studies with aequorin in squid axons indicate that cytoplasmic calcium activity goes up rapidly after mitochondrial ATP-dependent calcium uptake is

blocked by oligomycin (Baker, Hodgkin & Ridgeway, 1971). It is important to note that the same study found that an equally large portion of calcium buffering is independent of metabolic energy suggesting that calcium binding to proteins or membranes may be important. Equilibrium dialysis showed that these energy-independent sites have a capacity of about 40 umoles per kg axoplasm (about 10% of total axon calcium) and were half-saturated at calcium concentrations of 0.4 uM (Baker & Schlaepfer, 1975; Baker, 1976). Indeed, it is well known that calcium binds to proteins (Abood, Hong, Takeda & Tometsko, 1976; and to biological membranes (Cohen & Solomon, 1976; McDonald, Bruns & Jarrett, 1976) but there is no information at present on how important this is in intracellular calcium homeostasis.

The calcium-accumulating abilities of smooth endoplasmic reticulum in both skeletal and smooth muscles has been studied extensively using subcellular fractions obtained by differential centrifugation (Baudoin-Legros & Meyer, 1973; Hurwitz, Fitzpatrick, Debbas & Landon, 1973; Meisner, Conner & Fleischer, 1973). Similar fractions that exhibit ATP-dependent calcium uptake have been isolated from such non-muscle tissues as brain (De Meis, Rubin-Altschul & Machado, 1970), platelets (Robblee, Shepro & Belamarich, 1973), and liver (Moore, Chen, Knapp & Landon, 1975) and from secretory tissues such as the submaxillary and parotid salivary glands (Selinger, Naim & Lasser, 1970; Alonso, Bazerque, Arrigo & Tumlasi, 1971), neurohypophysis (Thorn, 1976), and pancreatic islets (Sehlin, 1976). The study of Moore et al. (1975) in the liver is of particular note because these workers (unlike some of the studies cited) carefully showed by enzyme markers that the calcium-accumulating microsomal fraction was completely free of plasma membrane contamination. To date no study has provided definitive evidence that smooth microsomal membranes regulate the free calcium activity in a secretory cell but the isolation of such calcium accumulating microsomal fractions indicates that this possibility must be considered.

While intracellular calcium buffering systems quickly absorb increases in cytoplasmic calcium they clearly do not have an infinate

capacity. Because all cells at rest have an inside negative membrane potential, calcium, if allowed to come to electrochemical equilibrium would be at a 50 to 400 fold higher activity in the cytosol than in the extracellular space. Since the extracellular medium of most cells has a calcium activity of about 1 mM while the intracellular activity is less than 1 uM this is clearly not the case. Although the calcium permeability of the plasma membrane is in most cases low (e.g. 1/1000th that of the sodium permeability in squid axon; see Mullins 1976) calcium is continually flowing into the cell down its electrochemical gradient and would eventually saturate all intracellular buffers if it were not transported out of the cell. Two methods for transporting calcium out of cells have been extensively studied. There is good evidence that calcium extrusion from red blood cells is accomplished by a Ca^{++}, Mg^{++}-dependent ATPase located in the plasma membrane which uses chemical energy from ATP hydrolysis to power calcium transport against a concentration gradient (Schatzmann & Vincenzi, 1969; Schatzmann & Rossi, 1973; Schatzmann, 1975). Calcium efflux in a number of tissues is known to be dependent on metabolic energy (e.g. liver (Van Rossum, 1970), L cells (Lamb & Lindsay, 1971), kidney (Moore, Fitzpatrick, Chen & Landon, 1974), and HeLa cells (Borle, 1969)) and suggests that the mechanism found in red blood cells may operate in other tissues as well. Nerve and muscle plasma membranes contain a 1:3 calcium-sodium countertransport system which uses sodium influx down its electrochemical gradient to power extrusion of calcium (Baker, 1972; Reuter, 1974; Blaustein, 1976). Variation of intraaxonal calcium activity with extracellular sodium concentration suggests that this transport system is a major determinant in maintenance of low cytoplasmic calcium activities in squid axon (Mullins, 1976). While it is not known whether all cells possess one of these two transport systems it is clear that all cells must possess some means of extruding calcium in order to maintain low levels of intracellular calcium.

It is apparent then that there is enough calcium either in the extracellular space or within cells themselves such that the

cytosol calcium activity could be increased several orders of magnitude if it were allowed to redistribute and reach equilibrium. It is also clear that potentially, alteration of any of the above mentioned mechanisms for keeping cytosolic calcium low could be used by the cell to momentarily raise the cytosol calcium activity and thereby create a calcium signal. These include: a) the plasma membrane permeability to calcium may be increased resulting in an increased influx of calcium down its electrochemical gradient; b) calcium may be released from intracellular organelles which sequester calcium such as mitochondria or smooth endoplasmic reticulum; c) calcium may be released from passive binding sites on membranes or proteins; and d) reduction in the amount of calcium pumped out of the cell could increase cytoplasmic calcium. There is now clear evidence that the first, an increased influx of extracellular calcium, is used in neural tissues to trigger release of neurotransmitters and hormones.

3. Stimulus-Secretion Coupling in Neural Tissues

Stimulus-secretion coupling was first studied in three neural secretory systems: release of acetylcholine or catecholamines from nerve terminal (Kuffler, 1944; De Castillo & Stark, 1952; Hukovic & Muscholl, 1962; Katz, 1962; Burn & Gibbons, 1964; Katz & Miledi, 1965, 1967; Boullin, 1967; Kirpekar & Misu, 1967; see Rubin, 1970, for a historical review), of catecholamines from the adrenal medulla (Douglas & Rubin, 1961, 1963; Douglas & Poisner, 1962; Douglas, 1968), and of vasopressin and oxytocin from the posterior pituitary (Douglas & Poisner, 1964a,b; Ishida, 1968). In all of these it was found that secretion initiated by depolarization or in the case of the chromaffin cell by acetylcholine was proportional to the extracellular calcium concentration (Douglas & Rubin, 1961, 1963; Douglas & Poisner, 1964a; Katz & Miledi, 1967; Ishida, 1968) and that removal of extracellular calcium completely abolished stimulation of secretion within a few

minutes (Douglas & Rubin, 1961; Douglas & Poisner, 1964b). Furthermore it could be demonstrated that triggering of secretion was accompanied by an increased uptake of extracellular calcium. Uptake of $^{45}Ca^{++}$ into slowly exchangable pools in both the posterior pituitary or adrenal medulla was increased by secretory stimulation (Douglas & Poisner, 1962, 1964b; Russell & Thorn, 1974; Borowitz, 1969) and the depolarization accompanying acetylcholine stimulation in the adrenal was found to be partially dependent on an inward calcium current (Douglas, Kanno & Sampson, 1967a,b). In addition it has been shown that introduction of extracellular calcium into the cell by ionophore is capable of stimulating secretion in cholinergic nerve terminals (Kita & Van Der Kloot, 1974, 1976), adrenal medulla (Cochrane, Douglas, Mouri & Nakazato, 1975; Garcia, Kirpekar & Prat, 1975; Hochman & Perlman, 1976), and posterior pituitary (Nakazato & Douglas, 1974; Russell, Hansen & Thorn, 1974). The most elegant evidence for calcium's role in neural secretion has come from studies with the presynaptic nerve terminal at the squid giant synapse. Miledi (1963) first showed that introduction of calcium directly into the cytoplasm of the terminal by iontophoresis stimulated transmitter release. Llinas and coworkers (Llinas & Nicholson, 1975) have used aequorin to show that the intracellular calcium activity is increased by single presynaptic spikes as well as repetitive electrical stimulation. The time course and voltage dependence of the calcium transients paralleled those for transmitter release and strongly suggested that such transients are the physiological signal for secretion in this cell. Using voltage clamping techniques, Llinas has demonstrated a potential-dependent inward calcium current in these terminals which is proportional to transmitter release measured simultaneously (Llinas, Steinberg & Walton, 1976).

4. Stimulus-Secretion Coupling in Non-Neural Tissues

In contrast to neural tissues which all seem to rely on an increased influx of extracellular calcium to increase cytoplasmic calcium activity, non-neural secretory tissues vary, some using extracellular, others intracellular, and still others a combination of these two sources of calcium to trigger secretion. Antigen induced release of histamine and serotonin from mast cells has been shown to be completely dependent on extracellular calcium (Foreman & Mongar, 1972) and is accompanied by an increased uptake of calcium (Foreman, Mongar & Gomperts, 1973). It is also clear that an increase in cytoplasmic calcium activity is a sufficient stimulus for secretion. Artificial introduction of calcium into the cell by either ionophore (Cochrane & Douglas, 1974; Kagayama & Douglas, 1974) or iontophoresis (Kanno, Cochrane & Douglas, 1973) results in immediate exocytosis of granules. Stimulation of histamine release by the synthetic polyamine 48/80 is not completely dependent on extracellular calcium. The fact that application of this compound to a single mast cell by iontophoresis elicits a localized secretory response (Diamant, Kruger & Uvnas, 1970) and that EDTA treatment for 30 minutes eliminates response to the secretagogue (Douglas, 1975) suggests that 48/80 releases intracellular calcium from the plasma membrane or a site nearby. Thus either extracellular or intracellular calcium may be used independently to trigger exocytosis, and which is used is stimulus dependent.

Release of enzymes and components of the vitilline membrane from cortical granules in echinoderm and teleost eggs at the moment of fertilization is also triggered by calcium. Introduction of calcium into eggs by application of divalent cation ionophores will initiate cortical granule release (Steinhardt & Epel, 1974). Spreads of cortical granules sitting on a plasma membrane (isolated by ripping the upper portion of the cell away from its bottom adhering to polyamine-coated glass) will undergo fusion when exposed to calcium (Vacquier, 1975, 1976). Studies using aequorin in the very large

Medaka egg (Ridgeway, Gilkey & Jaffe, 1976) demonstrate beautifully that there are extremely well localized increases of calcium activity inside the egg during fertilization. The calcium sensitive luminescence of aequorin was seen to originate at a single point (presumably that of sperm entry) and spread out in a circular wave moving over the surface of the sphere to again coalesce at a point opposite its origin. The ionophore A23187 produced similar patterns but with multiple origins. The response to both sperm and A23187 were unaffected by extracellular calcium and could be elicited in the presence of EDTA (Jaffe, 1976). In addition, a 20-fold increase of isotopic calcium efflux from slowly exchangable compartments is observed when A23187 is applied to sea urchin eggs (Steinhardt & Epel, 1974). These data suggest that release of cortical granules is triggered solely by release of calcium stored at a site near the plasma membrane.

In contrast to mast cells and eggs, which utilize calcium from extracellular and intracellular stores, respectively, the endocrine pancreas appears to use both to regulate or initiate insulin secretion. Glucose-stimulated insulin secretion requires extracellular calcium (Curry, Bennett & Grodsky, 1968; Milner & Hales, 1968, 1970) and is proportional to its concentration up to 2 mM (Curry et al., 1968). Stimulation is accompanied by an increased uptake of isotopic calcium from the extracellular medium (Hellman, Sehlin & Taljedal, 1971, 1976; Malaisse-Lagae & Malaisse, 1971) and calcium-dependent action potentials (Dean & Matthews, 1970a,b; Matthews, 1970; Pace & Price, 1972; Dean, Matthews & Sakamto, 1975; Matthews & Sakamoto, 1975) suggesting that glucose induces large inward calcium currents across the plasma membrane. Antagonists of calcium influx that are thought to reduce the calcium permeability of the plasma membrane such as verapamil (Devis, Somers, Van Obberghen & Malaisse, 1975), D600 (Malaisse, Devis, Pipeleers & Somers, 1976), and lanthanum (Hellman, 1975) inhibit insulin secretion. While these observation make it clear that extracellular calcium is of vital importance for insulin secretion other observations suggest that intracellular

calcium also plays a role. Glucose stimulation is known to produce a marked elevation in cyclic AMP content of islets (Charles, Fanska, Schmid, Forsham & Grodsky, 1973; Grill & Cerasi, 1974; Hellman, Indahl, Lernmark & Taljedal, 1974; Charles, Lawecki, Pictet & Grodsky, 1975). Because methylxanthines also raise islet cyclic AMP content (Charles et al, 1976) and stimulate release of calcium from slowly exchanging pools (Brisson & Malaisse, 1973) it has been suggested that the rise in cyclic AMP induced by glucose may release intracellular calcium (Brisson, Malaisse-Lagae & Malaisse, 1971; Cerasi, 1975). In fact, theophylline, in the absence of extracellular calcium does elicit a small release of insulin (Charles et al., 1976). Thus in the islet both extracellular and intracellular calcium may play a role in stimulus-secretion coupling.

Even from these few examples of non-neural secretory tissues it can be appreciated that secretory cells, like muscle cells, are diverse in the source of calcium used as an intracellular signal.

5. The Release Process

It has been demonstrated quite clearly in a number of tissues that the release process that calcium triggers is exocytosis. Electron micrographs of secretory tissues fixed during stimulation frequently display omega-shaped invaginations of the plasma membrane about the size of a secretory granule that are interpreted as the end product of fusion between the granule and plasma membrane. Such "exocytotic figures" have been seen in a number of tissues as catalogued by Poste and Allison (1973) with excellent examples published for exocrine (Palade, 1975) and endocrine pancreas (Orci, 1974), parotid (Amsterdam, Ohad & Schramm, 1969), neurohypophysis (Douglas, Nagasawa & Schulz, 1971), and mast cells (Lagunoff, 1973). In freeze fracture one can observe the lips of these invaginations and occasionally the interior of the cavity and its continuity with the plasma membrane (e.g. see Smith, Smith, Winkler & Ryan, 1973; Heuser,

Reese & Landis, 1974; Orci, 1974). Most convincing, however, is exocytosis in the mast cell. The core of mast cell secretory granules is dense and does not dissipate after extrusion from the cell thereby allowing one to record this process cinematographically (Douglas, 1974).

Corroborating this morphological evidence is secretory evidence showing that enzymes contained in granule membranes are not secreted whereas substances within the granule matrix, although varying widely in their chemical properties, are secreted in parallel (Winkler, Schneider, Rufener, Nakane & Hortnagl, 1974). For example, dopamine beta-hydroxylase, which is localized in the membranes of chromaffin granules, is not released (Viveros, Aqueros & Kirschner, 1969) whereas ATP (Douglas, Poisner & Rubin, 1965) , a nucleotide, and the chromogranins (Kirshner, Sage & Smith, 1967), proteins, which are all contained within the granule along with catecholamines (Winkler, 1976) are all secreted. These results are not consistant with the whole granule (i.e. membranes plus contents) being expelled from the cell nor with individual constituents being transported molecularly across the granule and plasma membranes in sucession. They can be accounted for by exocytosis -- fusion of the granule and plasma membranes to directly expose the granule contents to the extracellular space.

One of the great enigmas of the stimulus-secretion coupling scheme is how calcium acts to trigger exocytosis. Several hypothesis have been proposed which assert that calcium facilitates fusion of the granule and plasma membranes. This possibility is supported by observations in many vesicle and cell systems that membrane fusion is calcium dependent. Papahadjopoulos and coworkers (Papahadjopoulos, Poste, Schaeffer & Vail, 1974; Papahadjopoulos, Vail, Pangborn & Poste, 1976) used differential centrifugation and molecular sieve chromatography to quantitate fusion between single-walled liposomes composed of acidic phospholipids. They found that calcium promoted fusion although at fairly high concentrations, 10^{-3} to 10^{-2} M. Gratzl has used freeze fracture electron microscopy to quantitate fusion of

golgi-derived vesicles from rat liver (Gratzl & Dahl, 1976a,b), and of secretory vesicles for both beta cells (Dahl & Gratzl, 1976) and adrenal medulla (Dahl, Gratzl & Ekerdt, 1976). In all cases they observed a marked increase in the number of intervesicular fusions in the presence of low calcium concentrations, 10^{-7} to 10^{-6} M. This facilitation was specific for calcium; other divalent cations had no effect or else inhibited fusion. Similarly, Davis and Lazarus (1976) have reported an interaction of beta cell granules and plasma membrane (isolated from separate sources) which leads to release of insulin and is promoted by calcium at low concentrations. These authors also presented data for calcium promoted "secretion" from analogous granule-plasma membrane systems prepared from adrenal medulla and anterior pituitary. Cell to cell fusion during developmental processes may also be calcium dependent. Fusion of cultured myoblasts to form myotubes does not take place in the absence of extracellular calcium (Yaffe, 1970). In a preliminary report Schudt, Dahl and Gratzl (1976) reported that vesicles prepared from myoblast plasma membranes will fuse but only in the presence of calcium at extracellular concentrations, 10^{-4} to 10^{-3} M.

There are a number of possibilities as to how calcium could promote membrane fusion. Several authors have suggested that calcium allows secretory granules to approach the plasma membrane sufficently close to fuse. If two membranes each having a negative surface charge approach closely, a repulsive force due to the interaction of the electric double layers of these membranes could hinder further approach (Matthews, 1970). Divalent cations should be able to neutralize this surface charge and eliminate this repulsion. This theory, however, predicts that magnesium or calcium should work equally well since both divalent cations will neutralize the surface charge of isolated granules (Matthews, Evans & Dean, 1972; Dean & Matthews, 1974) and does not account for fusion being promoted specifically by calcium.

Others have favored the possibility that calcium promotes enzymatic modification of membranes such as formation of lysolecithin

by a phospholipase (Winkler, 1971; Bach, 1974). This is suggested by the fact that the phopholipase that generates lysolecithin does require calcium (Ferber, 1971), that lysolecithin does facilitate membrane fusion (Poole, Howell & Lucy, 1970; Howell, Fisher, Goodall, Verrender & Lucy, 1973), and that secretory granules of some cells (e.g. mast cells and chromaffin cells) do contain large amounts of this component (Smith & Winkler, 1968; Winkler et al., 1974). However, the level of phospholipase A varies greatly between different secretory cells (Ferber, 1971; Winkler et al., 1974) and the theory has the intrinsic problem of how to regulate lysolecithin action to produce localized fusion rather than cytolysis.

Recently attention has focused on the ability of calcium itself to modify the physical properties of membranes. Phospholipids in monolayers and bilayers are known to undergo a transition at a critical temperature below which they exist in a tightly packed, more ordered "solid" state and above which they are in a more random "fluid" state (Papahadjopoulos, Jacobson, Nir & Isac, 1973; Trauble & Eibl, 1974; Chapman, 1975). Calcium is known to raise this transition temperature by neutralizing the negative charge of acidic phospholipids and thereby stabilizing the more closely packed "solid" state (Trauble & Eibl, 1974; Jacobson & Papahadjopoulos, 1975). In isothermal systems, calcium can induce fluid to solid transitions in systems containing one phospholipid and can induce mixed phospholipid systems to separate into fluid and solid phases that coexist physically as shown by differential scanning calorimetry (Papahadjopoulos et al., 1974; Papahadjopolous et al, 1976) and spacially as shown by rapid freezing followed by freeze fracture electron microscopy (Papahadjopoulos et al., 1976). Thus calcium might be able to induce biological membranes to create solid and fluid microenvironments. The significance of this is that if the membranes are in a fluid state - as is required for membrane fusion whether it be between liposomes and cells (Martin & McDonald, 1976; Poste & Papahadjopoulos, 1976) or between cells (Papahadjopoulos, Poste & Schaeffer, 1973; Kosower, Kosower & Wegman, 1975) -- calcium could

create "islands" of "solid" phospholipids, that is, lateral phase separations. It is possible that the structural discontinuities present in phase separation might promote fusion. In this context, it is interesting to note that calcium both promotes fusion and produces phase separations in liposomes containing negatively charged phospholipids but not in those composed of neutral lipids (Papahadjopoulos et al., 1974, 1976a,b; Miller, Arvan, Telford & Racker, 1976; Miller & Racker, 1976).

Biological membranes are known to undergo temperature-induced fluid-solid phase transitions similar to those seen in pure phospholipid systems. Studies of bacterial membranes (Verkleij, Ververgaert, Van Deenen & Elbers, 1972; James & Branton, 1973; Rottem, Yashon, Ne'eman & Razin, 1973; Kleeman & McConnell, 1974; Verkleij & Ververgaert, 1975), isolated mitochondria (Hackenbrock, Hochli & Chan, 1976) and microsomal membranes from hepatocytes (Duppel & Dahl, 1976) which have used freeze fracturing in conjunction with spin labeling, differential scanning calorimetry or X-ray diffraction show that fluid to solid phase transitions (accomplished by lowering the temperature) are accompanied by reversible aggregation of intramembranous particles and formation of particle clear areas; this process is reversed by raising the temperature or in some cases by adding cholesterol - a molecule known to maintain fluidity of phospholipid monolayers and bilayers at low temperature. From analogous freeze fracture studies on liposomes containing reconstituted integral proteins (Chen & Hubbell, 1973; Grant & McConnell, 1974) it is thought that such "aggregation" could be an indication of lateral phase separations between smooth, particle-free regions that are rich in ordered (solid phase) phospholipids and particle dense regions that are still fluid (Hackenbrock et al., 1976). Whether similar phenomena can be induced by calcium or could play a role in exocytosis is not known. At this point one can only speculate whether movements of intramembranous particles and surface antigens away from the incipient site of exocytosis in mast cells (Chi, Lagunoff & Koehler, 1976; Lawson, Raff, Gomperts, Feutrell & Gilula, 1977) or enlargement of the rossette of

particles prior to mucocyst release in tetrahymona (Satir, Schooley & Satir, 1973) for example could reflect such localized changes in the fluidity of membranes of secretory cells.

B. Stimulus-Secretion Coupling in the Exocrine Pancreas

In the exocrine pancreas, stimulus-secretion coupling is initiated by the neurotransmitter acetylcholine (Hokin, 1950; Williams, 1975) or the gastrointestinal hormone cholecystokinin-pancreozymin (CCK-PZ)(Harper & Raper, 1943; Hokin & Hokin, 1956). Initiation of enzyme release by either secretagogue is accompanied by membrane depolarization, alterations in calcium uptake and efflux, changes in cyclic nucleotide levels and in addition is modulated by the extracellular calcium concentration. The role that each of these phenomena may have in initiating secretion will be discussed.

1. Receptor Interaction

Like other neurotransmiters and polypeptide hormones, acetylcholine and CCK-PZ are thought to initiate pancreatic secretion by interacting with a plasma membrane receptor. However, these receptors have not been characterized or their location firmly established. Iwatsuki and Petersen (1977) have shown that iontophoresis of acetylcholine just outside acinar cells results in rapid membrane depolarization, an effect that is known to accompany initiation of secretion, while iontophoresis of acetylcholine intracellularly has no effect. This can be interpreted as meaning that acetylcholine, in order to stimulate secretion must bind to a receptor available only on the outside of the plasma membrane. This receptor is probably analogous to muscarinic receptors in other tissues since atropine blocks all effects of cholinergic agonists on

the acinar cell. Presumably CCK-PZ binds at a different receptor since its actions are not blocked by atropine. Recently Galardy and Jamieson (1975) have prepared photoaffinity labels for this receptor that consist of a reactive aryl nitrene linked to pentagastrin, a peptide containing of the first five N-terminal amino acids of CCK-PZ. This label upon exposure with light covalently reacts with CCK-PZ binding sites and produces irreversible stimulation of enzyme release (Galardy & Jamieson, 1975). Use of this technique should enable localization and isolation of the binding molecule. Detection of CCK-PZ receptors using radioactively labled CCK-PZ analogues (e.g. H-caerulein) is underway (Deschodt-Lanckman, Robberecht, De Neef, Lammens & Christophe, 1976) and should allow pharmacological characterization of these sites.

2. Membrane Depolarization

The resting potential of acinar cells is about -40 mV inside negative (Dean & Matthews, 1972; Matthews & Petersen, 1973; Kanno, 1974). Application of cholinergic agonists or CCK-PZ depolarizes the cell about 12 to 14 mV (Matthews & Petersen, 1973; Matthews, Petersen & Williams, 1973; Nishiyama & Petersen, 1974; Greenwell, 1975; Poulsen & Williams, 1977a) and causes a decrease in input resistance (Nishiyama & Petersen, 1974, 1975; Petersen & Ueda, 1976a). The depolarization is dependent on extracellular sodium (Nishiyama & Petersen, 1975) suggesting that sodium influx is increased during stimulation. The secretagogue induced depolarization does not depend on extracellular calcium (Nishiyama & Petersen, 1975) except at high concentrations (88 mM) when it is depressed, suggesting that the depolarization contains no component due to an inward calcium current.

In contrast to neural tissues, membrane depolarization in acinar cells is not sufficient to initiate secretion. Raising extracellular potassium depolarizes acinar cells (Kanno, 1972; Matthews & Peteresen, 1973; Poulsen & Williams, 1977a) but such

depolarization results in neither plasma membrane resistance changes (Nishiyama & Petersen, 1974) or stimulation of enzyme release (Benz, Eckstein, Matthews & Williams, 1972; Argent, Case & Scratcherd, 1973; Poulsen & Williams, 1977a) if care is taken to include atropine to block the effects of acetylcholine released from endogenous stores. If the resting potential of the cell is lowered by K^+ in the presence of atropine, caerulein (a 13 amino acid polypeptide containing the active site of CCK-PZ) is able to stimulate amylase release in a normal manner while producing almost no further change in membrane potential (Poulsen & Williams, 1977b).

Thus the plasma membrane of acinar cells, unlike that of neural secretory cells does not contain voltage dependent calcium channels which can flood the cell with extracellular calcium and thereby initiate secretion. Depolarization and enzyme release are probably initiated by the same secretagogue-receptor interaction but depolarization appears to be a peripheral phenomenon that does not in itself lead to secretion.

3. The Calcium Requirement for Secretory Stimulation

When Hokin first studied enzyme release from the pancreas (Hokin, 1966) and found that presoaking pigeon pancreas slices in calcium-free, EDTA containing media completely abolished the secretory response to acetylcholine, he logically postulated that the pancreas, like the better studied neural tissues, used extracellular calcium as a trigger for enzyme release. Since then a number of observations have suggested that the role of calcium is considerably more complex.

Extracellular calcium modulates both resting and stimulated rates of enzyme release from the perfused pancreas (Kanno, 1972, 1976; Argent, Case & Scratcherd, 1973; Schulz, 1975) and from pancreatic fragments (Robberecht & Christophe, 1971; Heisler, Fast & Tenenhouse, 1972; Williams & Chandler, 1975). Stimulated amylase release increases as extracellular calcium is raised over the concentration

range of 1 to 5 mM in both preparations (Kanno, 1972, 1976; Heisler et al., 1972; Williams, unpublished observations). However, in contrast to Hokin's original conclusion, a number of studies have shown that triggering of secretion by CCK-PZ or cholinergic agonists is not abolished in the absence of extracellular calcium (Robberecht & Christophe, 1971; Heisler et al., 1972; Argent et al., 1973; Williams & Chandler, 1975; Kanno, 1976; Petersen & Ueda, 1976; Schreurs, Swarts, De Pont & Bonting, 1976a). In fact, if the calcium content of pancreatic fragments is reduced 50% by superfusion for 90 minutes in calcium free media, unstimulated and stimulated amylase release are lowered proportionally and the relative stimulation by bethanechol, a cholinergic agonist, is not reduced (Williams & Chandler, 1975). Furthermore, both resting and stimulated amylase release from dissociated acinar cells prepared from guinea pig pancreas is independent of extracellular calcium (Williams, Cary & Moffat, 1976). Several studies have shown that when EDTA is included in calcium-free media pancreatic stimulation is abolished (Argent et al., 1973; Case & Clausen, 1973); however, it is clear that this block does not occur until the pancreas has been exposed to EDTA for at least 60 minutes and therefore could be due to depletion of intracellular calcium rather than just chelation of the extracellular ion. Several investigators have clearly demonstrated cholinergic stimulation of enzyme release during shorter exposures to EDTA (Argent et al., 1973; Petersen & Ueda, 1976). Thus extracellular calcium does regulate enzyme release from the pancreas but, at least at the onset of secretion does not constitute a *sine qua non* for triggering of secretion.

Although the original conclusion that extracellular calcium is an absolute requirement for pancreatic secretion (Hokin, 1966; Benz et al., 1972) has since been disproven, a recent observation has strongly implicated calcium as the intracellular trigger for enzyme release. The divalent cation ionophore, A23187, in the presence of extracellular calcium will increase $^{45}Ca^{++}$ uptake (Williams & Lee, 1974; Kondo & Schulz, 1976a; Christophe, Frandsen, Conlon, Krishna &

Gardner, 1976) and stimulate amylase release (Selinger, Eimerl & Schramm, 1974; Williams & Lee, 1974; Scheurs et al., 1976a) from pancreatic acinar cells. In view of this ionophore's known ability to selectively increase the divalent cation permeability of membranes, this data suggests that A23187 increases influx of extracellular calcium into acinar cells and that the increased level of ionized calcium in the cytosol is able to trigger secretion.

How physiological secretagogues might initiate such a rise in cytosol calcium activity is a subject of current controversy and intensive study. Based on the modulatory effect of extracellular calcium on amylase release and on evidence of an increased influx of calcium during stimulation, some investigators feel that secretagogues act like A23187 to increase calcium influx (Kanno & Nishimura, 1976; Kondo & Schulz, 1976a,b) while others, based on evidence that secretagogues trigger release of isotopic calcium and on the fact that secretory triggering in not abolished by removal of extracellular calcium, have proposed that intracellular release of calcium is of primary importance for initiating secretion (Case & Clausen, 1973; Williams & Chandler, 1975). However, because no one has yet been able to measure cytoplasmic free calcium levels in acinar cells during secretion the relative importance of these two mechanisms for raising cytosol calcium levels is not known. Because there is data to support each view it may be useful to consider these not as opposing hypotheses but rather as separate facets of a stimulus-secretion coupling mechanism that may rely on more than one method of increasing cytosol calcium activity.

4. The Role(s) of Extracellular Calcium

a. Calcium Influx. In the now classic examples of stimulus-secretion coupling in neural systems (Douglas, 1968) secretion is triggered by increased intracellular free calcium which in turn is brought about by an increased influx of extracellular calcium across the plasma

membrane. Because of this precedent a large number of studies have been devoted to detecting whether pancreatic secretagogues stimulate calcium influx in acinar cells. Recently, Kondo and Schulz (1976a,b) have detected a 100 to 200% increase in $^{45}Ca^{++}$ uptake when either carbamylcholine or CCK-PZ is applied to isolated rat acinar cells. Secretagogue-induced uptake was observed as early as 3 minutes after addition of $^{45}Ca^{++}$ (Kondo & Schulz, 1976a) with uptake of tracer by both stimulated and unstimulated cells being dependent on the extracellular calcium concentration between 0.1 and 2.5 mM (Kondo & Schulz, 1976b). If this influx is important in triggering secretion, these characteristics could well account for the dependence of enzyme release on extracellular calcium and for the observation that bolus injections of calcium into the perfusion medium of an isolated pancreas results in rapid, transient bursts of enzyme secretion - an effect that is potentiated by the presence of submaximal levels of secretagogues (Argent et al., 1973; Schulz, 1975). These positive results, however, have not been confirmed by other laboratories. Gardner and coworkers (Gardner, Klaevemen, Adams & Ondetti, 1975; Christophe et al., 1976) also using isolated acinar cells (prepared from guinea pig rather than rat pancreas, however) measured comparable resting rates of $^{45}Ca^{++}$ uptake but could detect no stimulation of this rate by either carbamylcholine or a CCK-PZ analogue.

Similarly, most measurements of $^{45}Ca^{++}$ uptake in pancreatic fragments have detected no stimulated Ca^{++} influx (Case & Clausen, 1973; Williams & Chandler, 1975). In contrast, the techniques used in these studies were easily able to detect increased uptake of $^{45}Ca^{++}$ by either isolated cells (Christophe et al., 1976) or pancreatic fragments (Williams & Lee, 1974) when the divalent cation ionophore, A23187, and calcium were used to stimulate amylase release.

Alternatively, calcium uptake can be neasured by using La^{3+} to block efflux of intracellular $^{45}Ca^{++}$ while extracellular isotope is washed out - a technique developed by Van Breeman to detect uptake of calcium into cells in the presence of large amounts of calcium bound extracellularly (Van Breeman, Farinas, Casteels, Gerba, Wuytack &

Deth, 1973). Using this method, Heisler and Grondin (1973) reported that carbamylcholine did increase $^{45}Ca^{++}$ uptake into pancreatic fragments during the first 5 to 15 minutes of stimulation. However, Chandler and Williams (1974) in a more complete study of the effects of La^{3+} on $^{45}Ca^{++}$ uptake and efflux as well as amylase release could not detect any stimulation dependent entry of calcium by this same technique.

The basis of this discrepancy in uptake measurements is not known. One possible problem in comparing uptake data between fragments and isolated cells is that it is claimed that only 10% of the calcium in isolated cells is exchangable with $^{45}Ca^{++}$ (Kondo & Schulz, 1976a) while nearly all calcium is exchangable in pancreatic fragments (Case & Clausen, 1973; Clemente & Meldolesi, 1975b; Williams & Chandler, 1975). If it is true that only a small pool of calcium in isolated cells is exchangable (which seems questionable) singling out this pool for observation could be a distinct advantage if it is that used for triggering secretion; conversely, there is some danger that these are small fluctuations in size or accessibility of a larger pool that are not directly related to secretion.

As previously mentioned, electrophysiological studies have been unable to detect an increased inward calcium current during pancreatic stimulation (Nishiyama & Petersen, 1975). This is unlike neural secretory tissues such as the adrenal medulla (Douglas et al., 1967b) and the presynaptic nerve terminal of the giant squid synapse (Llinas et al., 1976) where an inward calcium current can be detected. However, in the case of the pancreas, it is possible that calcium influx could be electroneutral (as has been postulated for cardiac muscle; see Langer, 1976) or that the amount of calcium needed to activate secretion may be simply too small to detect.

Thus, it is clear that stimulation of calcium influx by pancreatic secretagogues is controversial at present. On the merit of several successful demonstrations it is possible that secretagogues stimulate uptake of extracellular calcium but this conclusion is tentative until confirmed by additional evidence.

b. Modulator of Release. Release of granule contents by exocytosis requires that the plasma and granule membranes fuse. It is not unreasonable then to consider that calcium binding sites on the exterior of the plasma membrane might modulate this process in addition to any regulatory effects calcium has intracellularly. Such modulation could account for the observation that both resting and stimulated amylase release from pancreatic fragments have a similar calcium dependence (Robberecht & Christophe, 1971; Heisler et al., 1972; Williams & Chandler, 1975). La^{3+}, which is thought not to enter cells but to displace Ca^{++} from binding sites on the exterior of cells, alters both resting and stimulated secretion in parallel; both are stimulated by La^{3+} at low concentrations and inhibited, then completely blocked by La^{3+} at higher concentrations (Chandler & Williams, 1974). Similarly, Kanno and Nishimura (1976) have reported that Mn^{++} competitively inhibits the ability of extracellular calcium to modulate amylase release but has little effect on a component of release that is similar in both time course and magnitude to release seen in the absence of extracellular calcium. All of these studies suggest that Ca^{++} acts at some external site to regulate resting and stimulated enzyme release. This calcium sensitive site is not the secretagogues receptor since dose-stimulation curves for bethanechol are identical in Ca^{++}-containing and Ca^{++}-free media (Williams & Chandler, 1975). The site could be either a calcium channel that determines calcium influx as suggested by Kanno (Kanno, 1972; Kanno & Nishimura, 1976) or a site modulating exocytosis; both equally well account for the above observations.

c. "Trigger" Calcium for Sustained Release. Although it is now clear that extracellular calcium is not absolutely required to trigger secretion when pancreatic secretagogues are initially applied there is good evidence that extracellular calcium is instrumental in maintaining enzyme output under sustained stimulation. In the absence of calcium, acetylcholine or CCK-PZ stimulate a rapid release of amylase in the first 10 to 20 minutes but continued stimulation

elicits progressively smaller amounts of amylase until nearly resting levels are reached (Heisler et al., 1972; Kanno & Nishimura, 1976; Petersen & Ueda, 1976b; Schreurs et al., 1976a). If calcium is then readmitted with continued presence of the stimulus, there is a large and immediate increase in enzyme release that is well maintained (Kanno & Nishimura, 1976; Petersen & Ueda, 1976b; Schreurs et al., 1976a). Because water and electrolyte secretion is relatively independent of extracellular calcium (Argent et al., 1973) the effect of calcium readdition is not due to "washout" of secretions from the duct system. There are two possibilities that could account for the effect. First, the acinar cell plasma membrane may become more permeable in Ca^{++}-free media thereby allowing the Ca^{++} readmitted to enter the cell and trigger secretion. A similar but much more pronounced release of catecholamines from the adrenal medulla upon calcium reintroduction has been accounted for in this manner (Douglas & Rubin, 1961; Douglas, 1968). This possibility is supported by the fact that acinar cells depolarize and exhibit a decreased membrane resistance in Ca^{++}-free media (Nishiyama & Petersen, 1975; Petersen & Ueda, 1976b) and that $^{45}Ca^{++}$ uptake by isolated acinar cells is increased by preincubation in Ca^{++}-free media (Kono & Schulz, 1976b). It would also explain the observation that reintroduction of calcium in some cases results in a transient increase in amylase release from superfused pancreatic fragments (Petersen & Ueda, 1976). However, the fact that reintroduction of extracellular Ca^{++} after a period of Ca^{++} deprivation elicits a five-fold greater release of amylase when acetylcholine is present (20 minutes preincubation) than under unstimulated conditions (compare Figs. 11 and 13 from Petersen & Ueda, 1976b) suggests that secretagogue action itself uses extracellular calcium in addition to any permeability changes experienced in Ca^{++}-free media under resting conditions.

A better explaination, it appears, is that pancreatic secretagogues, under conditions of sustained stimulation, use extracellular calcium either to trigger exocytosis directly, possibly by an increased influx of calcium, or indirectly to replenish some

intracellular compartment that acts as an initial source of trigger calcium but which is depleted during long term stimulation in Ca^{++}-free media. It is important to stress that most of the data demonstrating secretagogue-induced calcium influx in isolated acinar cells were obtained after 30 minutes stimulation (Kondo & Schulz, 1976a,b). Further study might reveal that the ability of secretagogues to promote calcium influx is small during initial application but increases with continued stimulation. The alternative, that some intracellular store or pool of calcium important for triggering exocytosis is depleted during sustained stimulation, is supported by the observation that repeated, pulsatile stimlulation with acetylcholine in Ca^{++} -free media elicits successively smaller amounts of amylase release while in Ca^{++}-containing media amylase release is not diminished for over 3 hours (Heisler et al., 1972; Argent et al., 1973; Petersen & Ueda, 1976b). However, amylase release after 1 to 2 hours of incubation in Ca^{++}-free medium is not markedly diminished if the gland is stimulated once rather than repeatedly or continuously (Heisler et al., 1972; Williams & Chandler, 1975). This suggestion is not unreasonable since calcium content of such intracellular organelles as mitochondria is known to depend on extracellular calcium levels (Borle, 1973). Thus it is possible that the pancreas uses extracellular calcium either as a source of trigger calcium during sustained stimulation or as a source of calcium to replenish the intracellular store of calcium that is responsible for triggering the initial component of amylase release that is independent of extracellular calcium. It should be pointed out that these mechanisms are not mutually exclusive. Cytoplasmic calcium activity is in the steady state, dependent on continual influx of extracellular calcium and continual removal by mitochondrial and plasma membrane transport systems. If secretagogues were to inhibit these transport systems, continued influx of extracellular calcium as well as any calcium release from internal stores could raise cytoplasmic free calcium and trigger secretion. In this case stimulation of amylase release would be dependent on extracellular

calcium levels but would not necessarily require an increase in the plasma membrane permeability to calcium.

5. The Role of Intracellular Calcium

If one considers that amylase release is triggered by a rise in cytosol calcium and that secretion can be initiated by secretagogues even in the absence of extracellular calcium, the conclusion that calcium must be released from internal stores is inescapable.

This possibility is supported by the observation that both acetylcholine and CCK-PZ rapidly increase the efflux of calcium from the tissue. If pancreatic fragments are loaded with $^{45}Ca^{++}$ then washed in isotope-free medium for 60 to 120 minutes (sufficient time to remove all extracellular and loosely bound isotope) application of either secretagogue increases the rate of $^{45}Ca^{++}$ efflux 4 to 6 fold for up to 20 minutes (Case & Clausen, 1973; Matthews, Petersen & Williams, 1973; Heisler, 1974; Schreurs et al., 1976a) while the efflux of other tracers such as $^{42}K^{+}$ (Case & Clausen, 1973) and $^{28}Mg^{++}$ (Schreurs et al., 1976b) remain relatively constant. The isotope release appears to be coming from an intracellular site because it is entirely unaffected by removal of extracellular calcium (Case & Clausen, 1973; Heisler, 1974; Williams & Chandler, 1975 ; Kondo & Schulz, 1976b; Schreurs et al., 1976a). It could be argued that this calcium release is a secondary effect of an increase in plasma membrane permeability. However, this is ruled out by the observation that in pancreatic fragments the ionophore A23187 stimulates amylase release by increasing the cell permeability to calcium but produces only a small increase in isotopic calcium washing out of slowly exhangable pools (Schreurs et al., 1976a; Williams, unpublished observation). Another alternative is that an increased influx of unlabeled calcium either displaces bound, isotopic calcium or is coupled to calcium efflux by a countertransport system. However,

neither of these explainations can account for efflux being stimulated in the absence of extracellular calcium. The only reasonable conclusion then is that pancreatic secretagogues release intracellularly bound or sequestered calcium from a pool that is only slowly exchangable and is relatively unaffected by the extracellular divalent cation concentration.

It seems more than a coincidence that the time course of this stimulated efflux is similar to the time course for amylase release in the absence of extracellular calcium for both the isolated, perfused pancreas and pancreatic fragments (Schreurs et al., 1976a; also compare secretory data in Kanno & Nishimura, 1976, and Petersen & Ueda, 1976b, with efflux data in Case & Clausen, 1973; Matthews et al., 1973; and in Williams & Chandler, 1975). In contrast, the time course for amylase release in the presence of extracellular calcium is not like that of the stimlulated efflux. Enzyme release usually has a sharp, initial peak which levels off to a steady level of stimulation that is maintained well beyond the time at which calcium efflux has returned to normal values (Matthews et al., 1973; Schreurs et al., 1975, 1976a; Kanno & Nishimura, 1976). These observations suggest that the release of intracellular calcium monitored by $^{45}Ca^{++}$ efflux is involved in triggering amylase release in the absence of extracellular calcium but that continued stimulation requires extracellular unlabled calcium either to replace the isotopic calcium released or directly to maintain cytosol calcium activity at a high level. This is consistant with the observation that the marked stimulation of amylase release initiated by readdition of extracellular calcium after sustained stimulation in calcium-free medium is not accompanied by an increased efflux of $^{45}Ca^{++}$ (Schreurs et al., 1975, 1976a).

6. The Source of Intracellular Calcium

The origin of the cell calcium released during stimulation is now known although the fact that it is slowly exchangable suggests that it is bound or sequestered. Additional evidence for this comes from experiments on isolated acinar cells which exhibit a stimulated calcium efflux similar to that seen in whole tissue (Gardner et al., 1975; Christophe et al., 1976; Kondo & Schulz, 1976b). Gardner & coworkers (Shelby, Gross, Lichty & Gardner, 1976) have detected a pool of isotopic calcium in these cells that is relatively tightly bound or sequestered (pragmatically defined as that $^{45}Ca^{++}$ which remained with cell membranes after the cells were lysed in a cold, hypotonic medium containing 10 mM EDTA, washed twice, and membranes and medium separated by filtration). The size of this "membrane bound" pool was shown to decrease 40% in the first five minutes of stimulation with either carbamylcholine or CCK-PZ octapeptide while unstimulated cells showed no change in pool size. Although these results give no indication of what this Ca^{++} pool is or why it is less tightly bound in the presence of stimulant, they are consistant with the hypothesis that some intracellular compartment is able to release calcium in response to secretagogues.

Meldolesi and coworkers (Ceccarelli, Clemente & Meldolesi, 1975; Clemente & Meldolesi, 1975a,b) Have used subcellular fractionation and ultrastructural methods to show that most calcium in pancreatic acinar cells is bound to smooth microsomes, zymogen granule membranes, mitochondria, and the plasmalemma. At present, the organelle that is the best candidate for releasing calcium is the mitochondrion. Clemente & Meldolesi (1975b) have shown that isotopic calcium in the mitochondrial fraction is decreased 50% in tissue stimulated with caerulein while that contained in other fractions is unchanged. Also using subcellular fractionation, Argent, Smith & Case (1976) found that both mitochondrial and light microsomal membrane fractions contain a divalent cation-stimulated ATPase but that only the mitochondrial fraction exhibited ability to accumulate $^{45}Ca^{++}$ from

the medium. Thus, the pancreas, unlike the submaxillary (Selinger et al., 1970; Alonso et al., 1971) and the parotid (Selinger et al., 1970) which posses a microsomal ATP-dependent calcium-accumulating system, appears to have no calcium-sequestering organelles other than mitochondria. However, one must make the proviso that a microsomal system may be present but has not been isolated in an undamaged state -- a realistic possibility for the acinar cell that contains large amounts of potentially proteolytic and lipolytic enzymes.

Other laboratories (Case & Clausen, 1973; Kondo & Schulz, 1976b; Williams, unpublished observations) have demonstrated that application of mitochondrial uncouplers or inhibitors of oxidative phosphorylation produces an increase in efflux of $^{45}Ca^{++}$ not unlike that seen during stimulation although no amylase is released, probably because secretion requires metabolic energy (Hokin, 1951; Baudoin, Colin & Dumont, 1969; Jamieson & Palade, 1971).

While it seems possible that mitochondria could be the organelle that releases intracellular calcium, any hypothesis that suggests release of calcium from an intracellular organelle must pose the question of how interaction of a secretagogue with a receptor at the plasma membrane is able to produce a signal that the calcium releasing organelle will act on. In the case of the mitochondrion two possibilities have been discussed: sodium influx or cyclic nucleotides. The first is that increased intracellular sodium may trigger release of mitochondrial calcium. Substance is given to this by the observation of Carafoli (Carafoli, Tiozzo, Lugli, Crovetti & Kratzung, 1974) that isolated heart mitochondria rapidly release calcium if as little as 5 mM NaCl is added to the extramitochondrial medium. In the exocrine pancreas, secretagogues are known to increase the Na^+ permeability of the plasma membrane (Nishiyama & Petersen, 1975) but it is not clear whether the resulting sodium influx is required for secretory triggering. Cholinergic stimulation of pancreatic fragments is inhibited in sodium-free media (Williams, 1975b); however, it has been argued that this is a "washout" phenomenon (Petersen & Ueda, 1976), that is, that the secreted enzymes

are not carried out of the duct systems because electrolyte and water secretion is negligible in the absence of sodium. This view is supported by the observation that stimulation of enzyme release from isolated acinar cells (which have no duct systems) is not affected by sodium removal (Williams et al., 1976). In addition intracellular concentrations of sodium and potassium calculated from sodium and potassium content and extracellular space measurements (Williams, 1975b) show no changes during stimulation, and intracellular potassium activity as measured by potassium-sensitive microelectrodes (J. H. Poulsen and B. Oakley, unpublished observations) is not changed. Increased sodium and potassium have been shown to release calcium from mitochondria isolated from pancreas (Argent et al., 1976) but only at very high concentrations (50 mM). At present then there is little evidence for sodium as an intracellular messenger.

7. A Role for Cyclic Nucleotides

Cyclic nucleotides may offer a mechanism whereby pancreatic secretagogues might signal intracellular organelles. In many cells receptor-mediated synthesis of cyclic AMP or GMP is followed by nucleotide activation of a protein kinase which in turn phosphorylates intracellular proteins and organelles.

The role of cyclic AMP in enzyme secretion by the exocrine pancreas is complicated by the fact that stimulation of electrolyte and water secretion by the gastrointestinal hormone secretin is mediated by this nucleotide. Secretin stimulation is accompanied by a rise in cyclic AMP content of the pancreas (Benz et al, 1972; Case, Johnson, Scratcherd & Sheratt, 1972; Robberecht, Deschodt-Lanckman, DeNeef, Borgent & Christophe, 1974; Deschodt-Lanckman, Robberecht, DeNeef, Labrie & Christophe, 1975) and application of dibutyryl cyclic AMP or methylxanthines either alone or in combination stimulates electrolyte and water output (Case & Scratcherd, 1972). Thus some investigators who have applied these agents to the perfused pancreas

or to pancreatic fragments may have observed increased digestive enzyme release due to "washout" of enzyme already present in the duct system. In addition, impure preparations of CCK-PZ may contain enough secretin to raise cyclic AMP levels. It is not clear whether these problems account for the fact that a number of laboratories have studied the effects of cyclic AMP on enzyme release but have reported remarkably inconsistant results. Several studies (Kulka & Sternlicht, 1968; Bauduin, Rochus, Vincent & Dumont, 1971; Beaudoin, Morris & Dunnigan, 1974) have reported that enzyme release is increased upon application of dibutyryl cyclic AMP and that this nucleotide potentiates the actions of calcium (Schulz, 1975) or secretagogues (Heisler et al., 1972). Other studies indicate that this nucleotide has no effect (Benz et al., 1972; Case & Schratcherd, 1972; Haig, 1974; Williams, 1974). Even in those studies which showed an effect of the nucleotide, cyclic AMP or its dibutyryl derivative produced effects no greater than 10 to 30% of those observed with normal secretagogues (Bauduin et al., 1971; Beaudoin et al., 1974). In addition, stimulation of enzyme release by CCK-PZ or cholinergic agonists is not accompanied by a rise in cyclic AMP content (Benz et al., 1972; Heisler, Grondin & Forget, 1974; Robberecht et al., 1974; Deschodt-Lanckman et al., 1975; Haymovits & Scheele, 1976) The cat pancreas, in vivo, exhibits cyclic AMP increases but these are seen even under conditions where no enzyme release occurs (Case et al, 1972). In addition, dibutyryl cyclic AMP has no effects on acinar cell membrane potential (Matthews & Petersen, 1973) or on calcium influx or efflux from these cells (Heisler, 1974; Williams, 1974; Kondo & Schulz, 1976b). Thus, cyclic AMP may have some indirect effects on enzyme release but most evidence indicates that it can not be the messenger which mediates the intracellular effects of CCK-PZ or acetylcholine. A particularly revealing series of experiments shows that application of cholera toxin, a molecule known to activate endogenous adenyl cyclase activity, to a cannulated rat pancreas (Kempen, De Pont & Bonting, 1975) or to a perfused cat pancreas (Smith & Case, 1975) increases cyclic AMP content and stimulates water and

electrolyte secretion markedly. At the same time, enzyme secretion was constant or reduced and the ability of CCK-PZ or acetylcholine to elicit enzyme release was not changed.

Cyclic GMP does, however, seem to be related to initiation of enzyme release. Studies both in vitro (Haymovits & Scheele, 1976; Christophe et al., 1976) and in vivo (Robberecht et al., 1974) demonstrate a 1 to 25 fold increase in pancreatic cyclic GMP levels within the first 2 to 4 minutes after stimulation of enzyme release by either CCK-PZ analogues or cholinergic agonists. Haymovits & Scheele (1976) have shown that the dose-response relationship for carbachol's ability to release amylase and increase cyclic GMP levels in guinea pig pancreas are identical. Also, Christophe et al. (1976) have shown in isolated acinar cells that the dose-response relationship for the ability of carbachol and 3 different CCK-PZ analogues (of differing molar potency) to stimulate Ca^{++} efflux and to increase cyclic GMP levels are identical. The same study showed that atropine blocked the ability of carbachol to increase cyclic GMP levels but not that of CCK-PZ, a result that parallels atropine's effects on amylase release. These data suggest that the secretagogue-induced rise in cyclic GMP, increase in Ca^{++} efflux, and stimulation of enzyme release are all initiated by agonist interaction at the same receptors. Whether these events are initiated sequentially or simultaneously is not yet known; both the rise in cyclic GMP (Haymovits & Scheele, 1976; Christophe et al., 1976) and the increase in calcium efflux (Case & Clausen, 1973; Matthews, Petersen & Williams, 1973; Williams & Chandler, 1975) are initiated and reach their maximum within 4 minutes--as least as early as the increase in amylase release (Matthews, Petersen& Williams, 1973). One possibility is that the rise in cyclic GMP levels is a consequence of the rise in intracellular free calcium. It is known that guanylate cyclases in membrane preparations from fibroblasts (Wallach & Pastan, 1976) and renal cortex (De Rubertis & Craven, 1976) are activated by calcium and that the ability of cholinergic agonists to increase cyclic GMP levels in many tissues is dependent on extracellular calcium (Schultz & Hardman, 1975). In the pancreas this

might be suggested by the observation that the divalent cation ionophore, A23187, also produces a sharp rise in cyclic GMP content (Christophe et al., 1976; Heisler, 1976). Conversely, could increased levels of cyclic GMP signal intracellular organelles to release calcium? It has been clearly demonstrated that cyclic AMP promotes calcium release from isolated mitochondria (Borle, 1974; Matlib & O'Brien, 1974) but similar actions for cyclic GMP have not been reported. Further studies will undoubtedly focus on whether cyclic GMP is an intracellular messenger that acts either to initiate release of intracellular calcium or to facilitate some step in exocytosis.

C. Detection of Calcium Redistribution Within Acinar Cells: The Aim of This Study

Although there is evidence that intracellular calcium is released during stimulation of the pancreatic acinar cell, and that calcium is able to trigger enzyme release, no one has been able to demonstrate an increase of ionized calcium in the cytosol or to localize the stores of calcium responsible for release. Calcium sensitive photoproteins such as aequorin have been used in large cells with spectacular success (Blinks et al., 1976; and section IA above) but this protein has not been introduced into cells as small as the acinar cell because pressure injection is required. Similarly, dyes sensitive to ionized calcium such as murexide (Scarpa, 1972) and arsenazo III (Di Polo et al., 1976) do not readily cross cell membranes making intracellular introduction of the dye difficult. In the last five years there has been much interest in using ultrastructural methods to localize intracellular calcium stores in part because it is now possible to detect the ions present in a semi-thin section of plastic embedded tissue with a resolution of a few microns by using electron probe analysis (Hall, 1971; Coleman & Terepka, 1974; Russ, 1974). However, tissue calcium must be immobilized by precipitation (Coleman & Terepka, 1972; Yarom, Peters,

Scripps & Rogel, 1974) or freezing (Davies & Erasmus, 1973; Heuser & Reese, 1976) prior to fixation and microscopy; precipitation techniques are often nonspecific (e.g. the pyroantimonte method) and are liable to artifactual redistribution of ions (Coleman & Terepka, 1972) while freezing techniques that avoid this are not generally available.

As an alternative, the present study attempts to detect a pool of bound or sequestered calcium that is released during pancreatic stimulation by using two probes whose fluorescence is divalent cation sensitive: the ionophore A23187 and the antibiotic chlorotetracycline. The use of these two probes differs in a fundamental way. A23187, because of its ionophoric properties, perturbs the normal distribution of intracellular Ca^{++}. Experiments with this probe were begun with the expectation that by defining its ability to stimulate amylase release and determining its subcellular distribution by fluorescence microscopy, we could make some conclusions about the presence of intracellular calcium stores able to trigger secretion by calcium release. In contrast, chlorotetracycline was used as a nonperturbing probe of calcium normally bound to membranes in acinar cells.

We applied these probes to pancreatic tissue enzymatically dispersed into single cells or small groups of cells. These preparations allowed probe fluorescence to be observed in living acinar cells by two techniques--fluorescence microscopy and fluorometry of cells suspended in a cuvette. The first method allowed histological localization of the fluorescence while the latter was suited to continuous monitoring of fluorescence during stimulation of secretion.

As previously noted, the ability of A23187 to stimulate secretion in the pancreas (Selinger et al., 1974; Williams & Lee 1974; Schreurs et al., 1976) as in a number of other tissues (Foreman, Mongar & Gomperts, 1973; Cochrane & Douglas, 1974; Russell, Hansen & Thorn, 1974; Charles et al., 1975; Cochrane et al., 1975; Garcia, Kirpekar & Prat, 1975; Wollheim, et al., 1975) has been thought to

mean that the ionophore acts at the plasma membrane to increase influx of extracellular Ca and that the resulting rise in cytoplasmic calcium activity is able to trigger exocytosis. The ionophore does increase calcium fluxes across the plasma membrane of whole cells (Reed & Lardy, 1972a; Foreman, Mongar & Gomperts, 1973; Williams & Lee, 1974; Christophe et al., 1976; Desmedt & Hainaut, 1976; Kondo & Schulz, 1976b; Reed, 1976) and at least two studies have used aequorin to show that levels of ionized calcium in the cytosol rise in the presence of A23187 and extracellular calcium (Desmedt & Hainaut, 1976; Rose & Loewenstein, 1976). However, the assumption that A23187 acts exclusively at the plasma membrane is probably incorrect. The fact that A23187 can release calcium from sarcoplasmic reticulum vesicles (Scarpa, Baldassare & Inesi, 1971; Caswell & Pressman, 1972), isolated mitochondria (Pressman, 1972; Reed & Lardy, 1972b; Wong et al., 1972; Binet & Volfin, 1975; Sordahl, 1975) and whole cells (Desmedt & Hainaut, 1976) and that A23187 induces secretion or contraction in some cells in the absence of extracellular calcium (Schroeder & Strickland, 1974; Steinhardt & Epel, 1974; Ashby & Speake, 1975; Charles et al., 1975; Karl et al., 1975; Murray, Reed & Fay, 1975) suggests that release of intracellular calcium is possible. In fact, Lardy and coworkers have shown that in sperm, A23187 has effects both at the plasma membrane and intracellularly at mitochondria (Babcock, First & Lardy, 1976). Thus, it was not clear whether A23187 acts on pancreatic acinar cells only by letting in extracellular calcium or whether it was also available at intracellular sites and able to release intracellular stores of calcium. In fact, no previous study had dealt directly with intracellular uptake of this ionophore.

In experiments presented in this thesis, fluorometry and fluorescence microscopy are used to demonstrate uptake by intracellular membranes of isolated acinar cells and to show that uptake is remarkably inhibited by the presence of extracellular divalent cations. A23187 stimulates amylase release from these cells at low concentrations and produces cell damage at higher concentrations; both actions are dependent on extracellular calcium.

Thus at the plasma membrane ionophore-mediated entry of calcium appears to initiate exocytosis and cytotoxic effects independently. In the absence of extracellular calcium (but in the presence of magnesium) A23187 does not stimulate amylase release, although we clearly show that it is available at intracellular membranes. For reasons that still are not apparent, A23187 does not appear to release intracellular calcium under these conditions.

Chlorotetracycline, used to detect redistribution of divalent cations normally bound to acinar cell membranes, is well known for its sensitivity to Ca^{++} and Mg^{++} in amphipathic environments. In the presence of erythrocyte and microsomal membranes (Caswell & Hutchison, 1971a; Hallett, Schneider & Carbone, 1972), detergent micelles (Caswell & Hutchison, 1971a), and alcohols (Caswell & Hutchison, 1971a; Caswell, 1972) this probe forms chelation complexes with Ca^{++} and Mg^{++} which fluoresce with 50 to 200 fold greater intensity than does CTC in aqueous solution. This fluorescence enhancement is dependent on the amount and type of divalent cations bound by the micelle or membrane (Caswell & Hutchison, 1971a). Circular dichroism and ultraviolet absorption spectra suggest that in amphipathic environments, CTC binds Ca^{++} with high affinity ($K_d = 9 \times 10^{-6}$) to form an adduct with a conformation unlike that of its Mg^{++} complex or its Ca^{++} complex in aqueous solution (Caswell & Hutchison, 1971b). This difference in conformation between Ca^{++} and Mg^{++} complexes is further born out in their fluorescence spectra in which the Ca^{++} adduct exhibits emission and excitation maxima at 530 nm and 398 nm, respectively, while those for the Mg^{++} adduct are shifted to 520 nm and 385 nm (Caswell, 1972; and section III of this thesis). These observations indicate that CTC fluorescence is sensitive to divalent cations associated with membranes and that the presence of Ca^{++} or Mg^{++} can be distinguished spectrally.

Fluorescence of CTC in mitochondria (Caswell & Hutchison, 1971a; Caswell, 1972; Schuster & Olson, 1974; Binet & Volfin, 1975; Luthra & Olson, 1976) and in sarcoplasmic reticulum vesicles (Caswell & Pressman, 1972; Caswell & Warren, 1972) has been shown to parallel

calcium movements produced in these organelles by substrates and inhibitors. As an example, CTC in mitochondria utilizing succinate as a substrate exhibit fluorescence of the Ca^{++}-CTC complex. Upon addition of an uncoupler, CTC fluorescence decreases in intensity and the remaining emission can be identified as being due to the Mg^{++} complex of CTC (Caswell, 1972). CTC has also been used as an approach to studying calcium in platelets (LeBreton, Dinerstein, Roth & Fineberg, 1976) and pancreatic islets (Taljedal, 1974) although the latter study did not provide spectral evidence that it was calcium being probed.

The present study shows that dissociated pancreatic acinar cells, loaded with CTC during preincubation, exhibit fluorescence that undergoes a remarkable intensity change upon stimulation with bethanechol or caerulein. To our knowledge this is the first study to use CTC to monitor stimulus-secretion coupling and to observe such secretagogue-induced fluorescence changes. We are able to show that CTC fluorescence in unstimulated cells is largely that of its Ca^{++} complex while fluorescence of stimulated cells is like that of its magnesium complex. Furthermore, we have obtained evidence that that portion of the CTC fluorescence affected by secretory stimulation is monitoring a pool of calcium which is influenced by mitochondrial inhibitors. This suggests that mitochondrial calcium may be redistributed during secretion.

II. MATERIALS AND METHODS

A. Preparation of Dissociated Pancreatic Acinar Cells and Other Cell Types

Buffers

Krebs-Henseleit Bicarbonate buffer (KHB) contained per liter (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.56; $MgCl_2$, 1.13; $NaHCO_3$, 25; $NaHPO_4$, 1.15; and was equilibrated with 95% O_2-5% CO_2 to give a pH of 7.35. Tris buffered Ringer's (TR) contained per liter (in mM): NaCl, 134; Tris HCl, 10; $CaCl_2$, 2.56; $MgCl_2$, 1.13; $NaHPO_4$, 1; KCl, 4.7; adjusted to a pH of 7.38 with NaOH and equilibrated with 100% O_2. Both KHB and TR contained 14 mM glucose, 1% bovine serum albumin (BSA, fraction V, Miles Laboratories), 0.1 mg/ml soybean trypsin inhibitor (Sigma Type 1-S), and Minimal Eagle's Medium amino acid supplement (GIBCO) except where noted.

Preparation of Isolated Pancreatic Acinar Cells

Isolated pancreatic acinar cells (see Fig. 10) were prepared by the method of Amsterdam and Jamieson (1972, 1974) with minor modifications (Williams, Cary & Moffat, 1976). Pancreatic tissue was obtained from male white Swiss mice (18 to 25 g) which were fasted 16 hours prior to use. Dissociation of the tissue involved two sequential digestions with collagenase-chymotrypsin with an interposed chelation of divalent cations by EDTA, and was completed by application of mild shearing forces. The KHB (or TR in a few experiments) used throughout the preparation had a magnesium concentration of 1.13 mM (except in the chelation step) but the calcium concentration was varied as indicated in the following procedural outline:

1. Eight to 12 mice were stunned, decapitated, and their pancreati excised, trimmed of small amounts of fat and connective tissue, and weighed. Usually, 0.7 to 1 g of tissue was used.

2. Five ml of an enzyme mixture (I) containing per ml 80 Units chromatographically purified collagenase, 0.09 mg ∝-chymotrypsin (both Worthington Biochemicals) and 1.5 mg hyaluronidase (Sigma Type 1) was injected into the interstitum of the pancreati through a 27 guage needle so as to distend them and rapidly expose the majority of lobules to enzymes.

3. The distended glands and excess enzyme solution were transferred to a 25 ml polycarbonate Erylenmeyer flask and incubated at 37° in a water bath shaking at 120 oscillations per minute. Incubation under these conditions was continued in steps 4 to 8 with the medium changed as designated.

Step	Medium	Volume	Ca^{++}	Length
3.	Enzyme Mixture I	5 ml	0.1 mM	10 min
4.	KHB containing 1 mM EDTA and no added Ca^{++} or Mg^{++}.	7 ml	10 M	4 min
5.	Repeat step 4.	"	"	"
6.	KHB	7 ml	0.1 mM	4 min
7.	Repeat step 6.	"	"	"
8.	Enzyme Mixture II (KHB containing per ml 120 Units pure collagenase, 0.1 mg chymotrypsin, and 1.8 mg hyaluronidase; enzyme concns. are higher than in Mixture I to compensate for medium trapped in the tissue).	5 ml	"	40-50 min

9. Soybean trypsin inhibitor (5 mg) was added to the flask and the tissue and medium sucked up and down 4 or 5 times with a 5 ml plastic pipette whose bore had been enlarged to about 2.2 mm in diameter. This was repeated using a 10 ml pipette with a 0.8 mm diameter bore. The resulting suspension was immediately filtered by gravity through a nylon stocking and a 70 micron mesh nylon cloth (Nytex) placed together (stocking on top) in a plastic funnel. This was followed by 10 ml of a rinse medium (KHB containing 1% BSA, Ca^{++} = 0.1 mM).

10. One-step gradients were prepared by layering 2 ml aliquots of the filtered cell suspension on top of 5 ml aliquots of KHB containing 4% BSA (Ca^{++} = 1 mM) in 105 x 12 mm polycarbonate test tubes. The gradients (six are needed) were centrifuged at 100 x g for 4 minutes to pellet the cells.

11. Cell pellets were resuspended, pooled, and washed 1 or 2 times in similar KHB containing 4% BSA (Ca^{++} = 1 mM). Cells were then resuspended in TR and preincubated for 1 to 2 hours.

At the begining of experiments cells were washed once and resuspended at a density of 1 to 3 mg dry wt per ml (equal to 0.55 to 1.65 mg protein per ml) in TR with the designated modifications. Unless noted otherwise preincubation and incubations for experiments were carried out in 25 ml siliconized glass flasks at 37° in a water bath shaken at 60 oscillations per minute.

Preparation of Dissociated Pancreatic Acini

Dissociated acini consisted of groups of 20 to 30 cells surrounding 1 or 2 lumens with individual cells retaining the polarity they possess in intact tissue. Acini were prepared in a manner identical to the method described above for isolated cells except that steps 4 through 7 were omitted and both enzyme mixtures I and II contained per ml 75 Units pure collagenase, 0.05 mg chymotrypsin, and 1.8 mg hyaluronidase. Acini were incubated in a manner identical to that for cells except that the medium for preincubation and all experiments with this preparation was TR with lower Ca^{++} and Mg^{++} concentrations: 1.28 and 0.56 mM respectively. This medium will be designated TR*. The divalent cation concentrations were lowered in order to reduce the tendency of the acini to aggregate and to increase the solubility of CTC; the divalent cation complexes of this probe only have limited solubility in aqueous media.

Preparation of Other Tissues

Isolated hepatocytes were prepared by the method of Seglen (1972) from livers of male Sprague-Dawley rats weighing 130 to 160 g.

Human blood (for fluorescence microscopy) was collected into heparinized tubes and used the same day. Whole blood was diluted 1 to 30 with TR and incubated as described above for dissociated acinar cells. Unsealed human erythrocytes (for fluorometry) were prepared by the method of Heinz and Hoffman (1965) using outdated blood bank blood.

Pieces of retina were obtained from dark adapted leopard frogs (Rana pipiens) and incubated at room temperature in the dark in TR diluted 7 to 9 with distilled water and equilibrated with 100% O_2. The incubated tissue was used directly or sliced into 100 micron sections with a Sorvall TC-2 tissue sectioner before microscopy. In one experiment retina pieces were incubated for one hour in buffer containing no calcium and 1.3 mg/ml crude collagenase (Worthington Biochemicals) in order to free individual photoreceptors.

B. Incubations and Assays

Measurement of Amylase, Lactate Dehydrogenase (LDH), or Fluorescent Probes Released into the Medium

Dissociated acinar cells or acini were incubated in 2 ml aliquots of TR or TR* with additions or deletions as noted. At the start of the incubation period, 1 ml of the cell suspension was centrifuged at 8000 x g for 20 seconds in an Eppendorf microcentrifuge (Brinkman Inst., #3200) and the supernatant assayed for amylase or LDH activity or for fluorescent probe content. The initial values (usually about 0.1 U/mg dry wt for amylase and 2% total for LDH) were subtracted from values obtained after incubation to obtain the enzyme activity or the amount of probe released during incubation. The

pellets of these initial samples were resuspended in 1 ml distilled water, transferred to a tared aluminum foil planchet, dried overnight at 80° and dry wt determined; alternatively, the pellets were sonicated in isotonic saline and the protein content assayed by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as a standard. Total cellular LDH activity was measured in a cell suspension frozen and thawed three tines during a 45 minute period. Amylase activity was measured by the method of Rinderknecht, Wilding and Haverback (1967) and is expressed in international units based on the reported activity of the amylase standard (Sigma type VI). LDH activity was measured spectrophotometrically by the method of Wacker, Ulmer and Vallee (1956). Fluorescent probes were assayed as described below.

Uptake of Chlorotetracycline

Dissociated acini were washed, then incubated in 7 ml of TR* containing 100 uM CTC but not BSA. At designated times, 0.7 ml aliquots were withdrawn, added to 8 ml of ice cold, isotonic saline and filtered with suction through a Nucleopore filter (3 u pore diameter). The filters, with acini, were placed in 2 ml of isotonic saline and sonicated for 10 seconds with a probe sonicator (as described below). Aliquots of the sonicate were assayed for protein by the method of Lowry et al. (1951) and for CTC as described below.

Assay of Chlorotetracycline (CTC), Oxytetracycline (OTC), and 1-Anilino-8-Naphthalenesulfonate (ANS)

To assay CTC or OTC a 200 ul aliquot of either sonicated acini or incubation medium was added to 800 ul of a solution containing 10 mM sodium dodecyl sulfate (Sigma, 95% pure) and 1.13 mM $MgCl_2$. Fluorescence was measured using excitation at 380 nm and read at 520 nm for CTC and 510 nm for OTC. The amount of CTC or OTC present in the assay was determined from standard curves which were

linear between 10^{-10} and 2 x 10^{-9} moles of CTC or OTC. The presence of Ca^{++} (1.28 mM) in the medium samples lowered the reading by 6%. In addition to its excellent sensitivity this assay offered the advantage that sonicated membranes had no effect on the assay (if the fluorescence of a membrane blank was subtracted) and were readily solubilized in the SDS reagent.

To assay ANS a 200 ul aliquot of incubation medium was added to 800 ul of ethanol (100%, Rossville Gold Shield, IMC Chemical Group) and fluorescence excited at 370 nm and read at 470 nm. ANS contained in the assay was determined from a standard curve which was linear between 10^{-10} and 2 x 10^{-9} moles ANS.

Fluorescent Probe Addition

A23187 was added to incubation media as a solution in ethanol such that the final ethanol concentration was 0.5% in most experiments. This concentration of ethanol produced small to negligible effects on amylase release; controls contained ethanol where noted. The A23187 used in these experiments was generously provided by Dr. Robert Hamill of Eli Lilly Co.

Chlorotetracycline HCl and oxytetracycline HCl were obtained fron the Nutritional Biochemical Corp. or from Sigma and ANS from the Eastman Chemical Co.; all were used without further purification. They were dissolved directly in the preincubation or incubation medium on the day of use. CTC was added prior to addition of calcium or magnesium to the buffer because this probe is only moderately soluble in divalent cation containing media. The methanol used as a solvent for recording spectra was reagent grade from Mallinckrodt and contained no fluorescent impurities.

C. Fluorometry

Fluorometric experiments were carried out with an Aminco-Bowman spectrofluorometer having a band pass of 15 nm. The instrument

was modified to allow stirring and temperature control of the cuvette contents. Uncorrected spectra were recorded on an Esterline-Angus strip chart recorder with the wavelength scale marked manually via the event marker. Quinine sulphate dihydrate (1 mg/liter 0.1 N H_2SO_4) was used to verify the wavelength settings for the excitation monochronometer at 254 and 352 nm. Uptake experiments were carried out in one of two ways. Whole cells or acini or erythrocyte membranes were added as a concentrated suspension in a 0.15 or 0.30 ml volume, via a syringe and 16 guage needle to a cuvette containing 3 ml of A23187 or CTC-containing medium at a temperature of 37° while monitoring the fluorescence intesity of the suspension on a strip chart recorder. Alternatively, cells, acini, or membranes were suspended in 3 ml of probe-containing medium, incubated in 25 ml siliconized flasks in a shaking water bath at 37° , and at designated times poured into a cuvette and the fluorescence intensity read.

The "concentrated" suspensions of cells or membranes added to the cuvette were prepared as follows. Whole cells or acini (3 to 6 mg dry wt) were washed once and resuspended in 0.15 ml of the cuvette medium minus the fluorescent probe. Concentrates of sonicated acinar cell membranes were prepared by suspending an equivalent amount of dissociated acinar cells or acini in 0.15 ml of 7 mM EDTA (pH 7.4) and sonicating for 10 minutes with a bath sonicator (Laboratory Supplies Co., Model #T-80-80-1-RS, 80kHz 80/320 watts) in the case of cells or for 10 seconds with a probe sonicator (Bronwill, Biosonik IV, set at 15, low scale) in the case of acini. Unsealed human erythrocyte membranes were suspended in 0.1 mM EDTA at a protein concentration of 2.8 mg/ml and 0.3 ml of this suspension added to the cuvette. The suspension medium for all fluorometric experiments was TR or TR* containing calcium, magnesium, and EDTA as indicated but without albumin in order to minimize background fluorescence. Medium for experiments with A23187 also lacked trypsin inhibitor and amino acids.

To observe fluorescence of acini during efflux of CTC, acini were preincubated 60 to 80 minutes in TR* containing 100 uM CTC and 1% BSA. The CTC-loaded acini were washed once and resuspended in 3 ml of

medium (calcium, magnesium, or EDTA present as indicated) containing no CTC or BSA and their fluorescence monitored continuously as described above.

D. Microscopy

Fluorescence, phase contrast, and darkfield microscopy were carried out with a Zeiss photomicroscope. Fluorescence microscopy utilized a 200 watt mercury vapor lamp and an oil immersion condenser. A23187 fluorescence was observed with a UG2 exciter filter and 410-650 bandpass barrier filter while CTC fluorescence was observed with a BG12 exciter filter and 500 nm cutoff filter. Darkfield and phase contrast microscopy used a tungsten lamp without filters. Incubated cells and medium were applied to a glass slide and covered with a glass cover slip. Cells were photographed using films with ASA ratings of 125 (Plus-X) or 400 (Tri-X, both Kodak) and exposure times for fluorescence images of 0.5 to 6 minutes (CTC) or 4 to 6 minutes (A23187). Initial film magnifications were 160x or 320x.

For light and electron microscopy dissociated acinar cells or acini were fixed by adding an equal volume of 0.08 M sodium cacodylate, pH 7.4, containing 1.5% glutaraldehyde and 1% paraformaldehyde. Cells ar acini were then resuspended in full strength fixative and pelleted by centrifugation for one minute at 8000 x g in an Eppendorf microcentrifuge. Pellets were stored overnight at 4°, postfixed in 2% OsO in 0.08 M sodium cacodylate buffer, rapidly dehydrated in ethanol and embedded in Araldite. Sections 0.5 micron thick were stained with 1% toluidine blue for light microscopy. Silver thin sections were doubly stained with uranyl acetate and lead citrate and viewed in a JEM-100B electron microscope at 60 kV.

Quantitation of the fraction of dead cells in thick sections of preparations which had been treated with A23187 was carried out by scoring all cells as "dead" or "healthy". Cells designated a "dead" were obviously vesiculated such as those indicated by arrows in the

section shown in Fig. 10B. For each experimental condition 2 to 8 thick sections were counted, representing 4000 to 8000 cells. Counting was done "blind" and repeated at least twice for each section; duplicate counts generally agreed to within 10 percent.

Trypan blue permeability was tested by mixing cells plus medium with an equal volume of 4% trypan blue in 0.9% NaCl and placing a drop of this suspension on a hemocytometer grid.

III. STUDIES WITH IONOPHORE A23187

A. Results

1. Excitation Spectra of A23187 in Ethanol and Tris Ringer's

It is well known that the fluorescence excitation spectrum of A23187 is sensitive to polarity of the medium and to chelation of divalent cations (Case, Vanderkooi & Scarpa, 1974; Pfieffer, Reed, & Lardy, 1974). Changes in position and intensity of peaks have been used to characterize ionophore interaction with mitochondria and sarcoplasmic reticular vesicles (Caswell & Pressman, 1972; Wong et al., 1973; Case et al., 1974), but corresponding studies with whole cells have not been reported. In order to study the interaction of A23187 with whole, dissociated pancreatic acinar cells we first examined the excitation spectra of A23187 in ethanol and in the Tris buffered Ringer (TR) used for cell suspension, as well as the effect of calcium on these spectra. A23187 fluorescence was monitored at 430 nm in all experiments since its emission spectrum consisted of one broad peak, 429-435 nm, that was relatively unaffected by the experimental condtions.

In ethanol the A23187 excitation spectrum (Fig. 1A, solid line) consisted of two peaks with maxima at 287 nm (peak I) and 378 nm (peak II). Upon addition of calcium (Fig. 1A, dashed line) peak I shifted from 287 to 303 nm, both peaks I and II decreased in intensity, and the ratio of the intensities (II/I) decreased from 5.25 to 2.4. These spectra for A23187 in ethanol are similar to these obtained by Pfeiffer et al. (1974).

The excitation spectrum of A23187 in Tris Ringer was considerably different. In EDTA TR, the spectrum consisted of one peak of low intensity at 335 nm (Fig. 1B, solid line; note change in ordinate scale and 15-fold increase in ionophore concentration). In the presence of calcium and magnesium, the spectrum contained two peaks at 308 and 372 nm (Fig. 1B, dashed line) and the intensity of

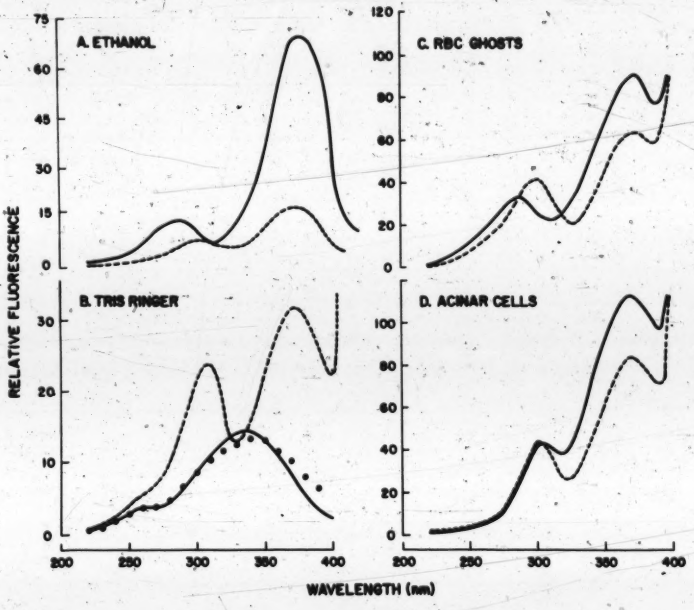


Fig. 1 A-D. Effect of calcium and magnesium on the fluorescence excitation spectrum of A23187 in ethanol, tris-buffered Ringer (TR), erythrocyte membranes, and dissociated pancreatic acinar cells. A. Spectra of 0.6 uM A23187 in ethanol with (----) or without (——) 2.56 mM $CaCl_2$ and 0.05% H_2O. B. Spectra of 10 uM A23187 in TR (----), in EDTA TR (—), or in TR which had been centrifuged at 27,000 x g for 5 minutes (....). The intensity of the supernatant spectrum only has been increased 33-fold for illustration purposes. C. Spectra of 10 uM A23187 in the presence of erythrocyte membranes. Spectra were recorded in either TR(----) or EDTA TR(——) 90 minutes after addition of membranes; final conc. 0.25 mg protein per ml. D. Spectra of 10 uM A23187 in pancreatic acinar cells. Acinar cells were incubated for 90 minutes at a cell density of 1.63 mg dry wt per ml in 3 ml of either TR (----) or EDTA TR (——) containing 10 uM A23187. Cells were washed once, resuspended in 3 ml of incubation medium minus ionophore, and their excitation spectra recorded. In all cases emission was monitored at 430 nm. Temperature was 30 . The baseline spectrum for buffer alone was arithmetically subtracted from Fig. 1B, ... but not from other spectra. Light scattering and fluorescence of erythrocyte membranes or acinar cells alone contribute no greater than 7.5 and 4 units, respectively, to spectra in 1C and 1D over the range 200 to 370 nm; the contribution increases substantially above 370 nm.

the spectrum was low compared to A23187 plus calcium in ethanol and was variable from preparation to preparation.

Further experiments suggested that the spectral characteristics of A23187 in the presence of calcium or magnesium could be attributed to A23187 being in the form of a particulate suspension. The fluorescence intensity of A23187 in TR was measured in control samples (Fig. 2, filled circles) and in the supernatant following centrifugation at 27,000 x g for 5 minutes (open circles). As shown in Fig. 2A, the intensity of A23187 in TR was decreased 60 to 98% by centrifugation. The excitation spectrum of the ionophore remaining after centrifugation (Fig. 1B, filled circles) was almost identical in form to that of ionophoare in EDTA TR (Fig. 1B, solid line). In contrast, the fluorescence intensity of A23187 in EDTA TR was decreased only 5 to 20% (Fig. 2B) by centrifugation and the form of its excitation spectrum (Fig. 1B, solid line) was unchanged. These data suggest that in the presence of calcium and magnesium A23187 is largely particulate and exhibits an excitation spectrum similar to that for ionophore in a less polar medium such as ethanol. Also present is a minor component, possibly ionophore in solution, having an excitation spectrum similar to ionophore in the absence of calcium or magnesium.

2. Spectra of A23187 in the Presence of Erythrocyte Membranes or Dissociated Pancreatic Acinar Cells

Addition of unsealed erythrocyte membranes to A23187 in EDTA TR resulted in the A23187 excitation spectrum changing from one peak at 335 nm (Fig. 1B, solid line) to two peaks at 288 and 372 nm (Fig. 1C, solid line). In the presence of calcium and magnesium, addition of erythrocyte membranes to A23187 produced a spectrum with two peaks at 302 and 372 nm (Fig. 1C, dashed line). These spectra were similar to those for A23187 in ethanol with respect to peak positions and alterations produced by divalent cations. In both cases the presence

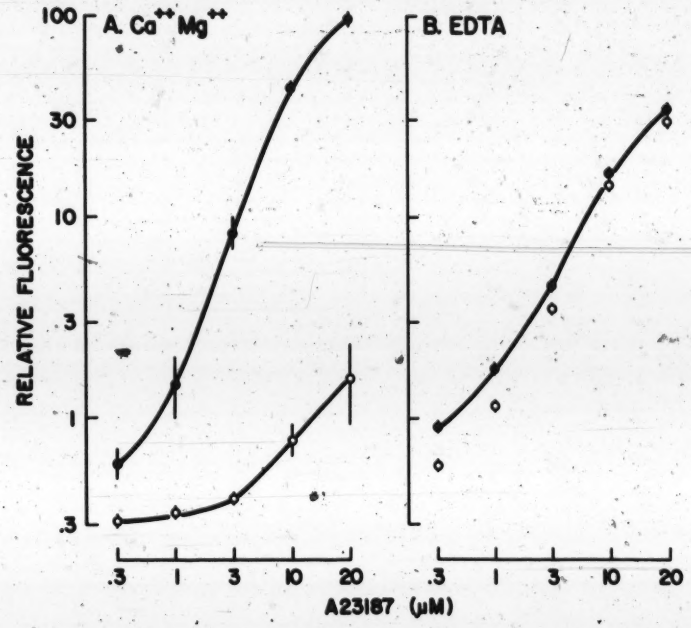


Fig. 2. Sedimentation of A23187 suspended in Tris buffered Ringer. Samples containing the designated ionophore concentration and 1% ethanol were prepared by adding stock solutions of A23187 in ethanol to 4 ml aliquots of TR (A) or EDTA TR (B). Samples were mixed by inversion, allowed to stand for 30 minutes at room temperature, mixed again, and divided into two groups. One group (○) was centrifuged at 27,000 x g for 5 minutes at room temperature and the other (●) left undisturbed. Following centrifugation, the fluorescence intensities of both groups were read. Fluorescence was excited at 370 (A) or 340 nm (B) and emission read at 430 nm. Symbols are means ± S.E. for 3 to 7 samples.

of calcium and magnesium resulted in a shift of peak I from 288 to 302 nm, and a decrease in the ratio of peak II to peak I intensity. (II/I changed from 5.25 to 2.4 in ethanol and from 3.06 to 1.4 in the presence of erythrocyte membranes). These spectra indicated that the greater portion of the fluorescence signal was from ionophore which had been transferred from the aqueous medium to the less polar environment of the erythrocyte membrane.

Spectra of A23187 taken up by dissociated pancreatic acinar cells (Fig. 1D) were measured after washing and resuspending the cells with ionophore-free media and represent fluorescence of cellularly associated A23187 with no component from ionophore in the extracellular medium. Excitation spectra exhibited two peaks, at 303 and 369 nm both in the presence and absence of calcium and magnesium. Again their resemblance to spectra for A23187 in ethanol suggested that the source of fluorescence was A23187 in a membrane environment.

3. Time Course of A23187 Uptake into Erythrocyte Membranes and Dissociated Acinar Cells

The striking changes in the excitation spectrum of A23187 upon its incorporation into membrane were used to study the time course of ionophore uptake by erythrocyte membranes and dissociated acinar cells. Figure 3A shows that when either acinar cells or erythrocyte membranes were added to 10 uM A23187 in EDTA TR, fluorescence intensity in response to optimal excitation at 310 or 290 nm, respectively, rose to a plateau with a half time of less then one minute. Membranes of acinar cells, although largely intracellular, took up ionophore almost as rapidly as the broken erythrocyte membranes. Separation of cells and medium after 5 minutes showed that by fluorescence less than 25% of the A23187 remained in the medium. In contrast, when acinar cells or erythrocyte membranes were added to 10 uM A23187 in TR containing calcium and magnesium, fluorescence intensity at 310 nm increased to a similar value but at a much slower

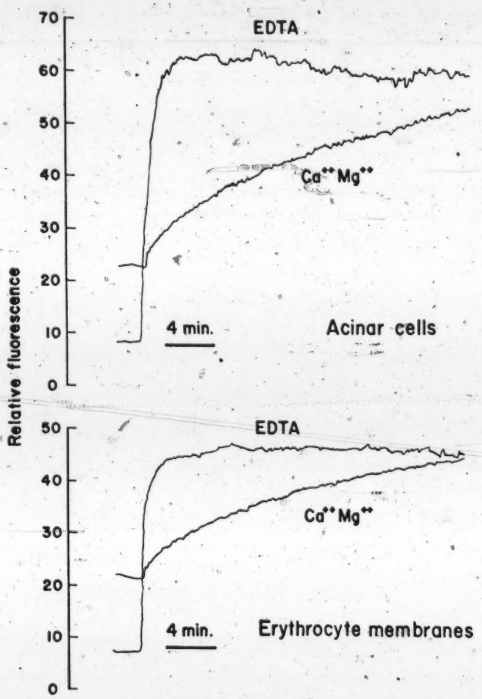


Fig. 3A. Kinetics of fluorescence intensity changes during uptake of A23187 by dissociated pancreatic acinar cells and unsealed erythrocyte membranes. Acinar cells or erythrocyte membranes (RBC ghosts) were added by syringe to a cuvette containing 10 uM A23187 in 3 ml of either TR (designated Ca^{++} Mg^{++}) or EDTA TR (designated EDTA). Final cell density was 1.70 mg dry wt per ml and erythrocyte membrane concentration was 0.25 mg protein per ml. Cuvette contents were stirred and maintained at 37°C. Fluorescence was excited at 310 nm except for erythrocyte membranes in EDTA TR where excitation was at 290 nm--the optimum for A23187 excitation under these conditions (see Fig. 1C, solid line). Emission was monitored continuously at 430 nm. The results are representative of 7 similar experiments.

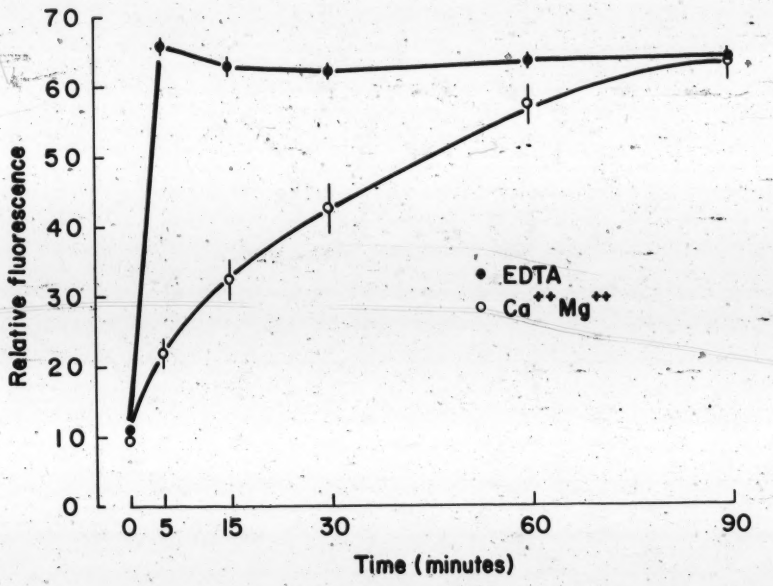


Fig. 3B. Time course of fluorescence intensity changes during uptake of A23187 by dissociated pancreatic acinar cells. Acinar cells were suspended at a cell density of 1.63 mg dry wt per ml in 25 ml flasks containing 10 uM A23187 in 3 ml of either TR (○) or EDTA TR (●). Flasks were incubated at 37° in a Dubnoff-type water bath and removed at the designated times to record fluorescence intensity. Fluorescence was excited at 310 nm and read at 430 nm. Symbols are means ± S.E. of triplicate incubations.

rate. Experiments in which fluorescence was monitored for longer periods (Fig. 3B) indicated that uptake by acinar cells required 20 minutes to become half maximal and reached a plateau only after 90 minutes. Centrifugation of A23187 (27,000 x g, 5 min) further reduced the rate of ionophore uptake into erytrocyte membranes by 90% suggesting that "particulate" A23187 contributed to uptake in the presence of divaleht cations.

4. Intracellular Localization of A23187 in Dissociated Acinar Cells

The excitation spectrum of A23187 incorporated into acinar cells was different from that of A23187 in ethanol or incorporated into erytrocyte membranes in one important aspect. Both the position and intensity of peak I were unaffected by the presence of calcium and magnesium in the medium (compare Fig. 1D with Figs. 1A and 1C). This suggested that A23187 associated with acinar cells may be intracellular and therefore not sensitive to extracellular ionic changes. This point was investigated further by comparing A23187 uptake into whole cells and sonicated cells. If A23187 is intracellular, disruption of whole cells by sonication should increase the exposure of ionophore to extracellular divalent cations. Such differences in exposure were looked for by incubating equal amounts of whole or sonicated cells with A23187 in the absence of divalent cations for 15 to 20 minutes, then adding magnesium. Magnesium rather than calcium was used to avoid triggering secretion. Figure 4 shows that uptake of A23187 from EDTA TR by both whole and sonicated cells was rapid and complete in five minutes. Addition of magnesium (final conc. = 1.13 mM) to a cuvette containing A23187 and sonicated cells produced an immediate and large increase in intensity of fluorescence excited at 310 nm. Excitation spectra show that addition of magnesium shifts the position of peak I from 302 to 308 nm and increases its intensity (compare solid and dashed spectra, Fig. 5). This shift is

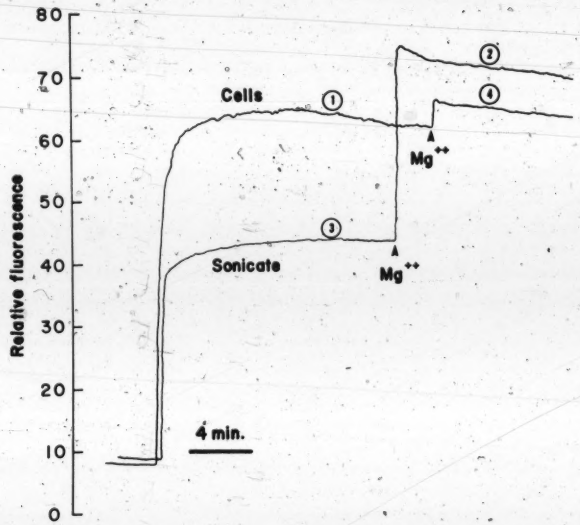


Fig. 4. Kinetics of fluorescence intensity changes during A23187 uptake by whole and sonicated pancreatic acinar cells in EDTA TR and subsequent addition of extracellular magnesium. Whole acinar cells or sonicated acinar cells were added by syringe to a cuvette containing 3 ml of 10 uM A23187 in EDTA TR. Final density of whole or sonicated cells was 1.67 mg dry wt per ml. Cuvette contents were stirred and maintained at 37° C. $MgCl_2$ (final conc. 1.13 mM) was added at the arrows. Fluorescence was excited at 310 nm and emission monitored at 430 nm continuously. The results are representative of six similar experiments.

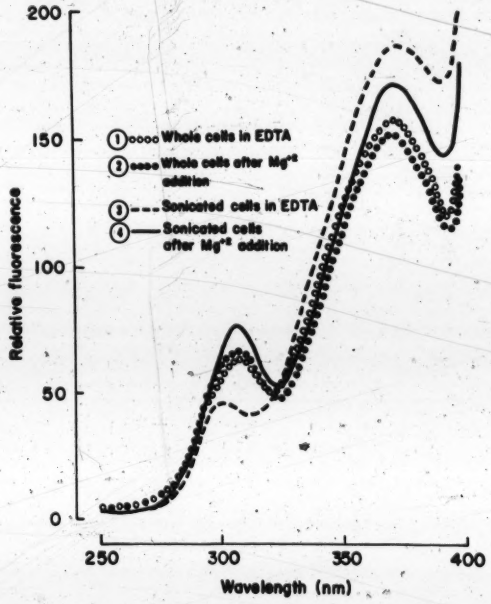


Fig. 5. Excitation spectra of A23187 incorporated into whole and sonicated pancreatic acinar cells in the absence and presence of extracellular magnesium. Excitation spectra were recorded during an experiment identical to that of Fig. 4 at the points indicated by circled numbers in Fig. 4.

similar, although not as large, as the shift of peak I in response to divalent cations seen in the spectra of A23187 in erytrocyte membranes (Fig. 1C). In contrast, addition of magnesium to A23187 in whole cells resulted in an immediate but much smaller increase in fluorescence intensity at 310 nm (see Fig. 4). Excitation spectra for A23187 in whole cells before and after addition of magnesium were nearly identical (compare open and closed circles in Fig. 4) and in fact were similar to the spectrum for A23187 in sonicated cells after magnesium addition. We interpret these changes in the excitation spectrum to mean that ionophore incorporated into membranes of broken acinar cells is exposed to extracellular ion changes and that peak I is a sensitive indicator of divalent cation chelation by membrane-associated ionophore. These changes are not due to transfer of A23187 from membrane to medium since this would increase the intensity of the entire spectrum. Rather, as shown in Fig. 5, peak I increased while peak II (373 nm) decreased in intensity. There was little or no contribution from ionophore not associated with membrane since identical spectral changes were observed with whole cells or sonicated cells which had been preincubated with A23187 then washed and resuspended in ionophore-free medium. The fact that the excitation spectrum of A23187 in whole cells was almost completely unaffected by magnesium addition and that this spectrum was similar to that for the ionophore in broken cells exposed to magnesium suggests that A23187 in whole cells is exposed to a finite but relatively constant divalent cation concentration in the face of large concentration changes in the extracellular medium. Because sonication resulted in spectral sensitivity of A23187 to extracellular cation addition, integrity of cell structure must be required to maintain this constant environment.

The protocol of the previous experiment (Fig. 4) was repeated for a twenty fold range of membrane concentrations. If ionophore incorporation were restricted to the plasma membrane of whole cells, an equal weight of cells broken up by sonication would present a many fold greater amount of membrane in which A23187 could be incorporated. Therefore, A23187 incorporation in sonicated cells should reach a

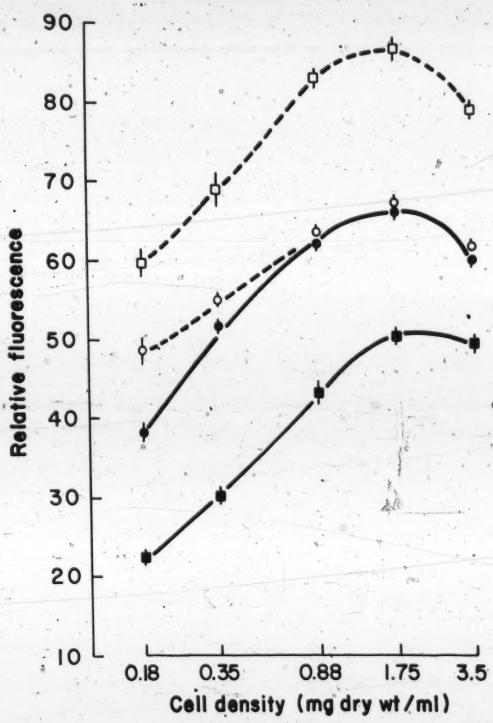


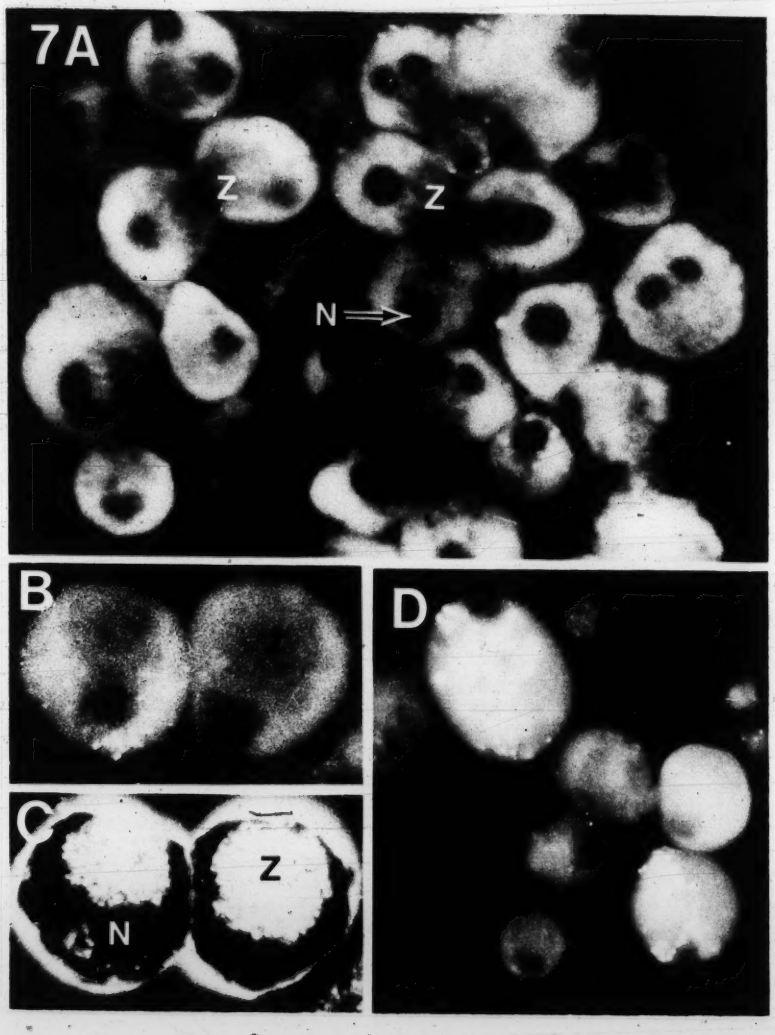
Fig. 6. Membrane concentration dependence of A23187 uptake by whole or sonicated pancreatic acinar cells. Whole or sonicated acinar cells were added to 25 ml flasks containing 10 uM A23187 in 3 ml of EDTA TR at the cell density (dry weight) specified. After 15 minutes incubation at 37°C in a Dubnoff-type water bath, the fluorescence intensity of cells and medium were recorded, $MgCl_2$ added (final conc. 1.13 mM), and fluorescence intensity read again 8 minutes later. Whole cells, before Mg^{++} addition = ●; after Mg^{++} addition = O; sonicated cells, before Mg^{++} addition = ■; after Mg^{++} addition = □. Data has been corrected for light scattering of cells in the absence of A32187. Symbols are means ± S.E. of triplicate incubations. The results are representative of two similar experiments.

maximum at considerably lower membrane concentrations than would ionophore interaction with whole cells. However, Figure 6 (filled squares and circles) shows that A23187 uptake by whole cells and uptake by sonicated cell membranes were similar in their dependence on membrane concentration: logarithmic functions of membrane concentration having similar slope in the range of 0.2 to 1 mg dry wt/ml, and reaching a maximum at 1.75 mg dry wt/ml. After addition of extracellular magnesium the fluorescence intensity of A23187 in sonicated cell membrane (open squares) increased dramatically at all membrane concentrations. The intensity of A23187 in whole cells (open circles) was unchanged by magnesium addition except at low membrane concentrations. We conclude that a similar amount of membrane is available for A23187 interaction in whole and sonicated cells.

5. Fluorescence Microscopy

Dissociated pancreatic acinar cells incubated in 10 uM A23187 displayed a blue fluorescence which as absent in cells not incubated with ionophore. Figure 7A shows that fluorescence was uniformly distributed in the cell with two exceptions. Nuclei (N) were not fluorescent and appeared as black "holes". Areas containing zymogen granules (Z) frequently emitted light of lower intensity. Positions of nuclei were confirmed by phase contrast microscopy and the positions of granular areas confirmed by phase contrast and darkfield microscopy. Figures 7B and 7C show fluorescence and darkfield images, respectively, of the same pair of cells. In Fig. 7C the zymogen granule areas (Z) scatter light intensely and correspond in position to areas of lower fluorescence intensity in Fig. 7B. As in Fig. 7A, the rest of the cells fluoresce except for the nuclear "holes". The uniform cytoplasmic fluorescence is observed within five minutes of incubation in 10 uM A23187 with calcium and magnesium either present or absent. When the medium contained calcium and magnesium large (1-5 u), highly fluorescent particles were present in the background, and

Fig. 7. Fluorescence and dark-field images of dissociated pancreatic acinar cells incubated with 10 uM A23187. A. Fluorescence of cells incubated 60 minutes with A23187 in OCa TR. N = nuclei, Z = zymogen granule areas. X 850. B and C. Fluorescence and dark-field images, respectively, of a pair of cells incubated 30 minutes with A23187 in OCaOMg TR. Z = zymogen granule area. X 1260. D. Fluorescence of cells incubated 45 minutes with A23187 in OCa TR. (Mg^{++} present.) X 865.


7A
Z
Z
N
B
C
Z
N
D

appeared to adhere to some cells. Other cells, such as those in Fig. 7D and the cell on the left in Fig. 7B, displayed uniformly small point sources of emission. This occurred frquently in 0Ca TR and only rarely in 0CaOMgTR. As suggested by Fig. 7D, a portion of the cell population often contained punctate fluorescence while neighboring cells had none. It was not possible to detect whether these sources of cellular emission were intracellular or extracellular. When 10 uM A23187 in TR was centrifuged at 27,000 x g for 5 minutes and then used to incubate cells, the cells again exhibited the pattern of emission seen in Fig. 7A. Although the fluorescence appeared within several minutes the intensity was low and there was a complete absence of particulate or pointlike sources in both cells and background. These observations are consistaent with the fluorometric data showing that medium fluorescence and medium uptake of A23187 is sharply reduced after removal of "particulate" A23187 by centrifugation.

6. Ionophore-Mediated Release of Amylase From Dissociated Pancreatic Acinar Cells: Calcium Dependence

A23187 (10 uM) in the presence of extracellular calcium and magnesium increased amylase release from dissociated pancreatic acinar cells by 201% ± 28% (mean ± S.E., n=14) during a 30 minute incubation. Figure 8 (compare filled circles and squares) shows that this ionophore-mediated release of amylase was well maintained over a two hour period with a 150 to 220% increase in release at each time point. In contrast, 10 uM A23187 in the absence of extracellular calcium and magnesium (but with magnesium present) had no effect on the rate of amylase release over the same two hour period (Fig. 8, compare open circles and squares) in 2 out of 3 experiments. In the third experiment, ionophore increased amylase release but only by 30% at 90 and 120 minutes.

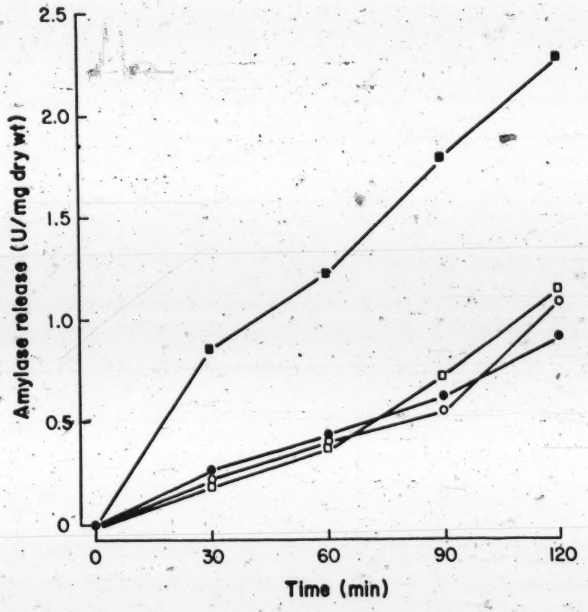


Fig. 8. Time course and calcium dependence of basal and A23187 stimulated amylase release from dissociated pancreatic acinar cells. Acinar cells were incubated at a density of 1.33 mg dry wt per ml in 4 ml of medium as follows: TR, no A23187 = ●; TR, 10 uM A23187 = ■; OCa TR, no A23187 = O; OCa TR, 10 uM A23187 = □. 0.5 ml aliquots of cells and medium were removed at the designated times and amylase release determined. Symbols are means of closely agreeing (within 10%) duplicate incubations. The results are representative of three similar experiments.

7. Ionophore-Mediated Cell Damage and Release of Lactate Dehydrogenase

Further experiments showed that cytoplasmic proteins such as lactate dehydrogense (LDH) as well as secretory proteins such as amylase were released by A23187. In the experiment shown in Fig. 9 (top) 10 uM A23187 increased amylase release by 414% in the presence of calcium but had almost no effect in the absence of calcium. In the same experiment, LDH release (Fig. 9 middle) was increased 740% in the presence of calcium. In the absence of extracellular calcium, LDH release was elevated, but the addition of ionophore had no additional effect. Comparable stimulation of amylase release (370%) by the cholinergic agonist bethanechol was not accompanied by increased LDH release. These data indicate that ionophore mediated LDH release in the presence of magnesium is calcium dependent and that such release is pathological in the sense that it does not occur during stimulation of secretion by normal secretagogues.

Because leakage of a cytoplasmic protein could result from compromise of the plasma membrane as a permeability barrier, ionophore mediated cell damage was further assessed by light and electron microscopic appearance of fixed cells and exclusion of the dye trypan blue by living cells. Structural damage was quantitated by light microscopy of cells incubated with and without ionophore for 30 minutes, then fixed, embedded and sectioned. Figure 10A shows a representative field of cells incubated in the absence of A23187. The cells are well stained, contain easily recognizable nuclei and zymogen granules, and the preparation contains only a small amount of debris. In contrast, Fig. 10B shows a typical field of a cell preparation which had been exposed to 10 uM A23187. Such preparations contained a large population of cells which were nearly normal in appearance and a smaller population of totally disrupted cells (arrows, Fig. 10B). Quantitation of disrupted cells (Fig. 9, bottom) showed that A23187 in the presence of extracellular calcium increased the dead cell count four-fold--from 2.2% in ethanol controls to 8.7%. In the absence of

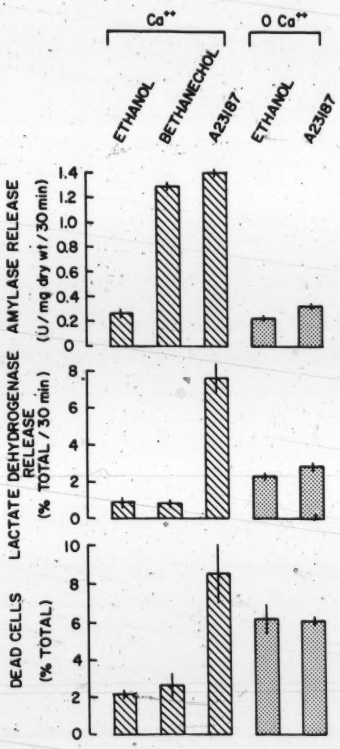


Fig. 9. Calcium dependence of amylase release, lactate dehydrogenase release, and structural damage produced by 10 uM A23187 in dissociated pancreatic acinar cells. Acinar cells, suspended at a density of 1.78 mg dry wt per ml, were incubated for 30 minutes in 1 ml of TR (hatched bars) or OCa TR (dotted bars) containing 0.5% ethanol, 150 uM bethanechol, 10 uM A23187 as designated. Cells and medium were then centrifuged and the medium assayed for amylase and LDH activities. Other cells, incubated in an identical manner, were fixed, embedded, and sectioned and "dead" cells quantitated by the manner described in the Methods section. Amylase and LDH activities are means ± S.E. of triplicate incubations from a single experiment representative of three similar experiments. Values for "dead" cells are means ± S.E. of four experiments.

Fig. 10. A. Representative field of dissociated pancreatic acinar cells incubated 30 minutes in TR. B. Representative field of acinar cells incubated 30 minutes in TR containing 10 uM A23187. Arrows denote cells scored as "dead." Both A and B, X 665.

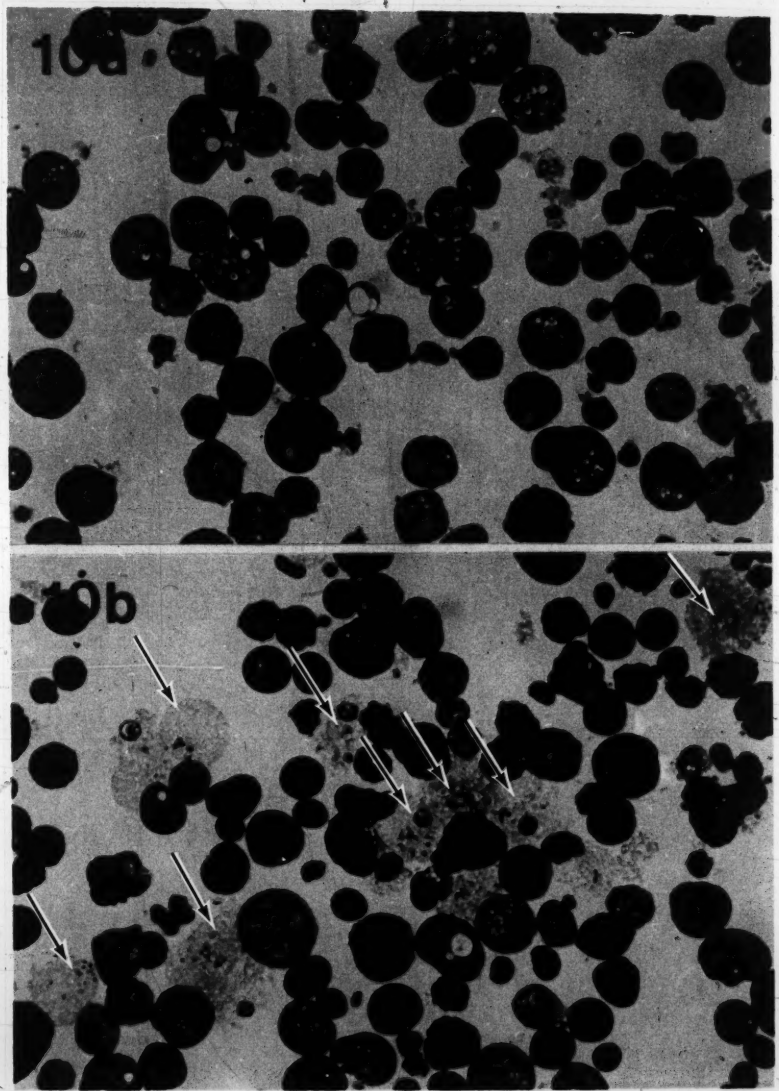
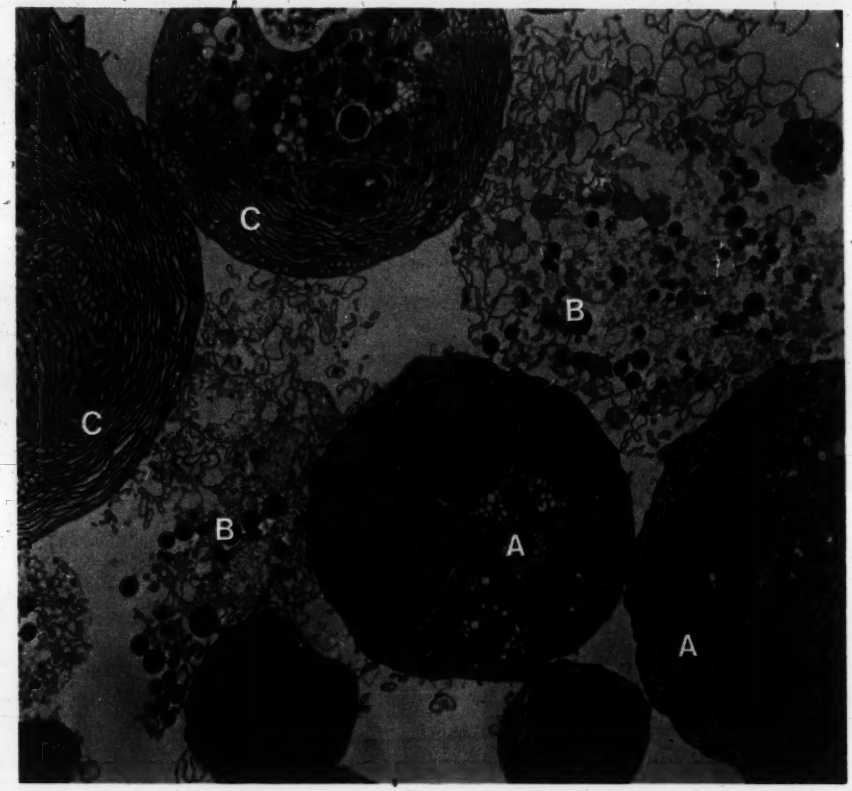

10a
10b

Fig. 11. Ultrastructure of dissociated pancreatic acinar cells exposed to 10 uM A23187 in TR for 30 minutes. A = normal cells, B = totally disrupted cells, C = swollen and vacuolated cells. X 3500.



calcium the count was elevated to 6.2% but ionophore had no additional effect. The cholinergic stimulant, bethanechol, produced no increase in disrupted cells. In two experiments, trypan blue exclusion by live cells gave results consistent with those from light microscopy of fixed cells. Only 2% of control cells failed to exclude the dye. In the presence of 10 uM A23187 and calcium, 15 to 20% of cells became permeable to the dye.

Ultrastructure of cells exposed to 10 uM A23187 ranged from relatively normal (Fig. 11, cells labeled A) to the completely disrupted cells previously observed by light microscopy (Fig. 11, cells labeled B). Disrupted cells consisted of zymogen granules and swollen mitochondria randomly spaced in a matrix of vesiculated membrane. A larger proportion (30 to 50%) of intact cells exhibited swollen endoplasmic reticulum and/or large vesicles in the Golgi area (Fig. 11, cells labeled C). Although control cells often contained some vacuoles, the high frequency and great extent of cell swelling in the presence of ionophore suggested that these were intermediate stages of cell disruption.

Therefore, we conclude that 10 uM A23187 in the presence of extracellular calcium produces structural damage in a portion of the acinar cell population ranging from leakiness to total disruption. LDH release, trypan blue exclusion, and light microscopy all indicate that when magnesium is present such damage requires extracellular calcium and that damage is not produced by stimulation of amylase secretion by the cholinergic agonist bethanechol.

8. Differentiation of Amylase Release Due to Cell Damage and Calcium Influx

Complete structural disruption of at least a portion of the cell population by 10 uM A23187 suggested that amylase release by A23187 could result from cell damage as well as triggering of normal secretory processes by influx of extracellular calcium. To

differentiate between these two pathways, ionophore provoked amylase and LDH release were compared in their dose-response relationship and time course. Fig. 12 shows that the dose-response relationship for release of amylase and LDH by A23187 differed substantially. Amylase release was increased by lower concentrations of ionophore than was LDH release. A23187, at 1 uM, increased amylase release 140% without increasing LDH release while at higher concentrations (3 and 10 uM) A23187 increased release of both amylase and LDH.

Amylase and LDH release by A23187 also differed in time course during the first 30 minutes. As shown in Fig. 13, amylase release in the presence of 1 uM A23187 (open triangles) was increased 158, 154, and 83% over control values (filled circles), at 5, 10, and 30 minutes, respectively. In contrast, LDH release was identical to control values at each time point. Although 10 uM A23187 (Fig. 12, open squares) released both amylase and LDH, these actions greatly differed in extent during the first 5 minutes of incubation. At this time, amylase release increased 268% (mean of two experiments) while LDH release increased only 25% (mean of two experiments).

We conclude, on the basis of the dose-response relationship and time course, that A23187 is able to release amylase by an action other than cell damage--most likely by increasing cytoplasmic calcium activity through increased influx of extracellular calcium.

9. Ionophore-Mediated Amylase Release in the Absence of Extracellular Calcium and Magnesium

A23187, 10 uM, increased amylase release from dissociated pancreatic acinar cells 136 ± 27% (mean ± S.E., n=4) in the absence of both calcium and magnesium in the extracellular medium (Table 1). However, the ionophore also increased LDH release 189% ± 15% (mean ± S.E., n=3) under these conditions. Further experiments (not shown) indicated that ionophore-mediated amylase and LDH release in the absence of both calcium and magnesium could not be distinguished on

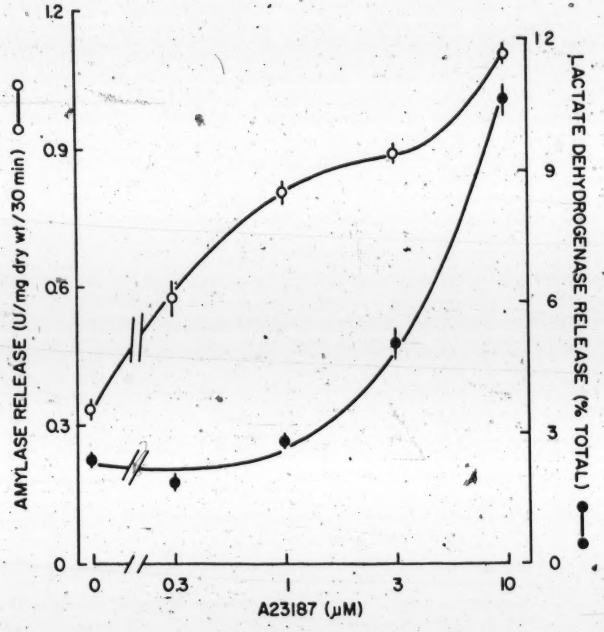


Fig. 12. Dose-response relationship for amylase and lactate dehydrogenase releasing actions of A23187 on dissociated pancreatic acinar cells. Acinar cells were incubated for 30 minutes in 2 ml of TR containing the designated concentration of A23187. Cells and medium were then centrifuged and assayed for amylase and LDH activities. Symbols are means ± S.E. of triplicate incubations. Results are representative of three similar experiments.

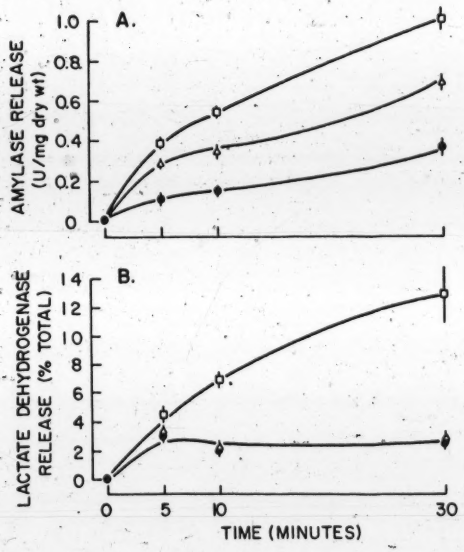


Fig. 13. Time course of amylase and lactate dehydrogenase release from dissociated pancreatic acinar cells by A23187. Acinar cells were incubated for the designated length of time in 2 ml of TR containing 0.5% ethanol (●), 1 uM A23187 (△), or 10 uM A23187 (□). Cells and medium were then centrifuged and assayed for amylase and LDH activities. Symbols are means ± S.E. of triplicate incubations. Results are representative of two similar experiments.

TABLE 1

AMYLASE AND LACTATE DEHYDROGENASE RELEASE FROM DISSOCIATED PANCREATIC ACINAR CELLS IN OCaOMg TRIS BUFFERED RINGER

Additions To Medium	Amylase Release (U/mg dry wt/30 min)	LDH Release (% Total/30 min)
Ethanol, 0.5%	0.46 ± 0.04 (4)	2.9 ± 0.25 (3)
A23187, 10 uM	1.10 ± 0.04 (4)	8.4 ± 0.88 (3)
Bethanechol, 300 uM	1.32 (2)	2.6 (1)

Acinar cells were incubated for 30 minutes in 2 ml of OCaOMg TR containing 0.1 mM EGTA and ethanol, A23187, or bethanechol as designated. Cells and medium were centrifuged and medium assayed for amylase and LDH activities. Values are means ± S.E. for the number of experiments shown in parentheses.

the basis of their dose-response relationships; both increased as the A23187 concentration was raised from 0.3 to 3 uM.

B. Discussion

This section considers questions which are fundamental to the use of the divalent cation ionophore A23187 in secretory tissues. Is A23187 taken up intracellularly? Do its uptake kinetics bear a relationship to its actions? Does release of secretory protein by ionophore and physiological secretagogues occur by the same pathway?

Our results indicate that A23187 is taken up intracellularly. Fluorescence microscopy shows that A23187 emission is distributed throughout the cell, but punctuated by the negative images of nuclei and zymogen granule areas. This emission pattern resembles the distribution of intracellular membrane. The acinar cell is packed with endoplasmic reticular, Golgi, and mitochondrial membrane except for the nucleus and zymogen granules. The latter are bounded by membrane, but contain large volumes of protein, nucleic acids, salts, and water which could account for their nonfluorescence. Negative contrast of internal organelles is not observed when the fluorescence is restricted to the surface of cells as exemplified by fluorescent antibody or concanavalin A binding to lymphocytes (Unanue, Perkins & Karnovsky, 1972; De Petris, 1975).

Fluorometric data also point to an intracellular location for A23187. The excitation spectrum of A23187 associated with intact cells (but not sonicated cells) indicates that the ionophore is exposed to a finite concentration of divalent cations which is relatively unaffected by changes in extracellular magnesium concentration. These results can be explained by the hypothesis that the majority of A23187 is incorporated into intracellular membrane which is exposed to cytoplasm having a magnesium activity of about 1 mM and that the integrity of the plasma membrane is required for maintenance of this concentration. Intracellular uptake of A23187 by

intact cells also accounts for the observation that disruption of cell structure by sonication does not increase the amount of membrane available for interaction with A23187 (see Fig. 6).

The effect of extracellular calcium and magnesium on the kinetics of A23187 uptake is striking. The rate of uptake is reduced twenty-fold in the presence of these ions. The rate-limiting step probably comes at or before the plasma membrane in whole cells since rate of A23187 incorporation into erythrocyte membranes which are completely exposed to the medium is also reduced by the presence of divalent cations. In addition, breaking of intact cells by sonication greatly increases the rate and extent of A23187 incorporation in the presence of calcium and magnesium (Chandler, unpublished observation). These observations also suggest that divalent cation effects on the kinetics of A23187 uptake are not due to A23187-mediated cell damage. Additional results making this unlikely are that A23187 in the presence of magnesium alone does not cause cell damage yet its uptake kinetics are similar to those observed when both calcium and magnesium are present. In the absence of calcium and magnesium (EDTA present) these cells maintain a permeability barrier to EDTA since intracellular A23187 is still complexed to divalent cations.

Although the effect of divalent cations on A23187 uptake is clear the underlying mechanism is not obvious. The heterogeneous physical state of A23187 in divalent cation containing media makes it possible that A23187 uptake occurs by more than one route. The sedimentation and microscopic observations presented suggest that the bulk of ionophore is present as particles up to 5 u in diameter and spectral data indicate that these particles coexist with a much lower concentration of soluble ionophore. The intense punctate fluorescence seen in a portion of the cell population (see Fig. 7D) may represent either A23187 particles taken up directly, for example by phagocytosis or simple adhesion, or soluble ionophore taken up and then concentrated by an intracellular organelle such as mitochondria. The diffuse fluorescence seen in the membrane-filled areas of acinar cells (see Fig. 7A) can be attributed to uptake of soluble ionophore. Such

a diffuse fluorescence pattern is seen in the virtual absence of the punctate pattern when soluble ionophore is taken up under two different circumstances--from EDTA TR and from regular TR which has been centrifuged at 27,000 x g to remove particulate A23187.

Fluorometric measurements show that divalent cations must limit the uptake of soluble ionophore since the changes in fluorescence intensity of A23187 that are monitored are a result of incorporation of ionophore molecules into the hydrophobic interior of membranes. Uptake could be limited by the rate that A23187 particles dissolve to form soluble ionophore. In fact, the observation that removal of A23187 particles by centrifugation reduces the rate of ionophore uptake into erythrocyte membranes by 90% suggests that particulate A23187 does act as a reservoir for soluble ionophore. Of greater interest is that A23187 uptake by erythrocyte membrane even after removal of particulate by centrifugation is slow (time of half-maximal uptake greater than 15 minutes). This is not due to the low ionophore concentration since A23187 at concentrations as low as 10^{-6} M is taken up rapidly from EDTA TR. It suggests that chelation with calcium or magnesium per se reduces the rate of A23187 incorporation into membrane.

These divalent cation effects on the time course of A23187 uptake may, in some cases, determine the time course of ionophore actions. Schreurs et al. (1976a) have shown that application of A23187 to fragments of rabbit pancreas in KHB results in a 100% increase in rate of amylase release but only after a lag of 30 minutes. In contrast, when fragments were superfused with A23187 in the absence of both calcium and magnesium and calcium then readmitted, amylase release was increased 300% within several minutes. Comparable results have been obtained in mouse pancreatic fragments (Poulsen & Williams, 1977a). The much faster uptake of A23187 in the absence of divalent cations may also account for the fact that in some tissues, notably posterior pituitary, addition of ionophore to a complete medium does not produce secretion (see discussion after Selinger et al., 1974) while preincubation with A23187 in the absence of calcium

and magnesium followed by calcium readmission produces an immediate, large release of secretory product (Russell et al., 1974).

It is also clear, however, that some actions of A23187 are immediate even if the ionophore is added to a divalent cation containing medium. Data in this report show that amylase release from isolated cells is increased 268% within 5 minutes. In pancreatic fragments membrane depolarization of surface cells occurs within minutes of A32187 application (Poulsen & Williams, 1977a). Similarly, other actions such as calcium uptake and triggering of exocytosis in mast cells (Mongar et al., 1973; Cochrane & Douglas, 1974), contraction of isolated smooth muscle cells (Murray et al., 1975), activation of sea urchin eggs (Steinhardt & Epel, 1974), and insulin release by perifused islets (Karl et al., 1975) are initiated within seconds to minutes of ionophore addition. Because almost all of these rapid actions involve isolated cells or superficially located cells, it is tempting to speculate that the availability of ionophore to interior cells of tissue fragments is limited by physical exclusion of ionophore particles as well as slower membrane uptake of chelated A23187.

Data presented in this report show that ionophore-induced release of secretory protein by a nonphysiological process is a real possibility. The increased release of lactate dehydrogenase (LDH), increased permeability to the dye trypan blue, and disruption of cell morphology produced by high A23187 concentrations (10 uM) all suggest that amylase could be released from structurally compromised cells. Although cells isolated by enzymatic hydrolysis may be more fragile than cells in intact tissues, reexamination of previously published data (Selinger et al., 1974) indicates the A23187 also increased LDH release from pancreatic fragments. Evidence of a different nature suggests that A23187 can release norepinephrine from adrenergic nerve terminals by a nonexocytic process. In vas deferens, Thoa and coworkers (Thoa, Costa, Moss, and Kopin, 1974) have shown that 100 uM A23187 released deaminated metabolites of norepinephrine as well as norepinephrine. This suggests that part of the norepinephrine was

released into the cytoplasm and metabolized rather than being released directly to the extracellular space via exocytosis. These results emphasize that one must interpret release of secretory products by high concentrations of A23187 with caution. This is particularly true in view of the fact that ionophore -m ediated cell damage (in the presence of magnesium) is calcium dependent just as are the secretory processes one is likely to be studying.

The ionic dependence of A23187-mediated cell damage requires that there be at least two mechanisms of action. That A23187 in the presence of magnesium requires extracellular calcium to produce cell damage (see Fig. 9) suggests that increased calcium influx is responsible. That A23187 also causes comparable cell damage in the absence of divalent cations (see Table 1) suggests that loss or redistribution of intracellular divalent cations or a direct membrane action of the ionophore may contribute to cell damage. Though the mode of action of ionophore in the absence of divalent cations is not clear it is remarkable that damage can be entirely prevented by the presence of extracellular magnesium.

The present study by ascertaining the time course and dose-response relationship of amylase release has been able to show that 1 uM A23187 plus extracellular calcium increases amylase release from dissociated acinar cells without producing cell damage. When extracellular calcium is absent but magnesium present, there is no evidence that the ionophore produces amylase release even after 2 hours. This is in spite of the fact that the ionophore is available intracellularly as shown by fluorescence microscopy and that uptake of ionophore reaches a plateau within 90 minutes. This could mean that the ionophore does not release intracellular calcium under these conditions. This conclusion is supported by the observation that application of A23187 to fragments of rabbit pancreas in KHB lacking calcium and magnesium does not noticeably increase efflux of calcium from slowly exchanging pools (Schreurs et al., 1976).

This study has also shown that A23187 increases amylase release in the absence of both calcium and magnesium--an observation

of potentially great interest since physiologic secretagogues can cause release of cellular calcium and trigger secretion in the absence of extracellular calcium (Williams & Chandler, 1975; Williams et al., 1976). In this case, however, amylase release and LDH release induced by A23187 cannot be differentiated on the basis of the dose-response relationship. Therefore, amylase release could be an artifact resulting from cell damage and further interpretation is unadvisable.

The usefulness of A23187 as a tool for increasing intracellular calcium lies in its ease of application. At present, the only alternative is iontophoretic application of calcium ions through an intracellular microelectrode--a technique that is limited to large, whole cells and whose results can be technically difficult to observe. However, use of A23187, though technically simple, has its own complexities. Uptake of ionophore and kinetics of its actions may be dependent on whether divalent cations are present during application. Calcium-dependent release of secretory protein resulting from cell damage is possible. Finally, the insolubility of A23187 in divalent cation containing media poses a real difficulty in determining what the effective concentration of ionophore is and by what route it is being delivered to cell membranes. While these findings do not invalidate the use of A23187, they suggest that care must be taken in the interpretation of such data.

IV. STUDIES WITH CHLOROTETRACYCLINE

A. Results

1. Chlorotetracycline (CTC) Does Not Alter Secretion From Pancreatic Acini

Because CTC at very high concentrations is known to inhibit a number of enzymes and biological precesses such as protein synthesis and oxidative phosphorylation (Laskin, 1967) it was necessary to ascertain that this probe does not interfere with enzyme secretion by pancreatic acini. Acini were exposed to CTC in one of two ways. In efflux experiments, acini were preincubated for 60 minutes in TR* with 100 uM CTC and 1% BSA, then washed and resuspended in medium containing no CTC or BSA. These are referred to as "CTC-loaded" acini. In uptake experiments, acini were preincubated in TR* containing BSA but no CTC, then washed and resuspended in TR containing 100 uM CTC but no BSA at the start of the experiment. Exposure to CTC in either protocol had no effect on cholinergic stimulation of amylase release from dissociated acini as shown in Table 2. In each case the amylase released from control and CTC incubated acini was identical with the exception that unstimulated release is lowered during CTC uptake. In each case bethanechol was able to increase amylase release 350% over unstimulated values. In a separate series of experiments isolated pancreatic acinar cells displayed normal secretory stimulation and normal ultrastructure after 100 minutes of incubation in 100 uM CTC (see Fig. 30). These results suggested that CTC could be used as a fluorescent probe with no gross disturbance to the normal secretory processes in acinar cells.

TABLE 2

EFFECT OF CHLOROTETRACYCLINE (CTC) ON AMYLASE RELEASE FROM UNSTIMULATED AND BETHANECHOL-STIMULATED PANCREATIC ACINI

CTC Additions		Amylase Release (U/30 min/mg protein)	
Preinc.	Incub.	Control	Bethanechol (100 uM)
NONE	NONE	1.00 ± 0.10	4.28 ± 0.33
CTC	NONE	0.87 ± 0.04	4.06 ± 0.18
NONE	CTC	0.46 ± 0.02	4.10 ± 0.31

Dissociated pancreatic acini were preincubated 60 minutes in TR* containing 1% BSA, then washed and resuspended in TR* containing no BSA and incubated 30 minutes during which time amylase released into the medium was measured. The preincubation or incubation medium contained 100 uM CTC as indicated; the incubation medium contained 100 uM bethanechol as indicated. Values are means ± S.E. of triplicate incubations. Results are representative of two similar experiments.

2. Fluorescence Changes in CTC-Loaded Acini During Stimulation

Acini, preloaded with CTC, resuspended in CTC-free medium, then placed in a fluorometer cuvette, exhibited a fluorescence which decreased wtih time. As shown in Fig. 14A, the intensity decreased faster in the first five minutes and more slowly but consistently thereafter. Addition of 100 uM bethanechol during the slow phase (see Fig. 14B) resulted in a marked loss of fluorescence occurring mainly in the first six minutes of secretagogue action. After this time fluorescence intensity continued to decrease at a rate similar to that before bethanechol addition.

A similar decrease in fluorescence (Fig. 14C) was seen upon application of caerulein, a ten-amino-acid polypeptide containing the active site of CCK-PZ. The drop in intensity was generally more abrupt with caerulein than with bethanechol; this was probably not due to diffusional limitations since higher concentrations of bethanechol (300 uM) did not result in a more rapid loss of fluorescence.. As shown in Fig. 14D, atropine completely inhibited the bethanechol induced fluorescence change but did not affect that resulting from caerulein stimulation. These affects parallel atropine's ability to inhibit bethanechol-stimulated but not caerulein-stimulated amylase release and suggest that the fluorescence decrease, like secretion, is initiated by bethanechol acting at a cholinergic receptor or by caerulein acting at another receptor that is not blocked by atropine.

Because stimulation by either bethanechol or caerulein in intact pancreas is accompanied by depolarization we tested whether depolarization itself could produce the fluorescence changes seen during stimulation. As shown in Fig. 14E, increase of the extracellular potassium concentration from 4.7 to 58 mM--a concentration known to substantially depolarize acinar cells (Matthews & Petersen, 1973)--had no effect on the fluorescence of CTC-loaded acini while bethanechol added 4 minutes later had its usual effect.

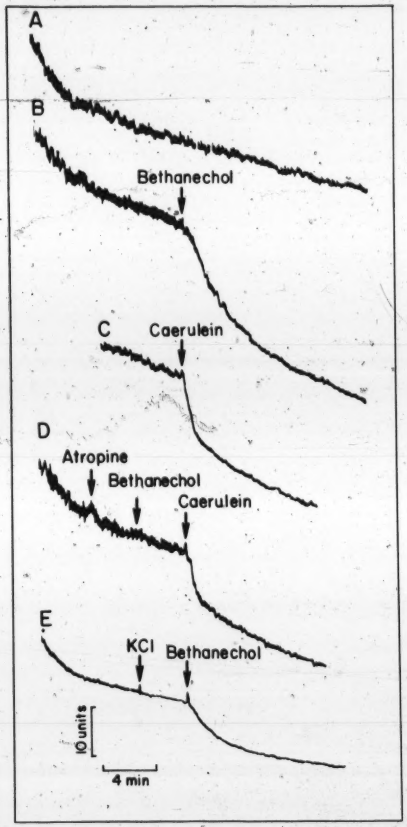


Fig. 14. Effect of bethanechol, caerulein, atropine and KCl on fluorescence of CTC-loaded pancreatic acini. Acini were preincubated for 60 to 80 minutes in TR* containing 100 uM CTC and 1% BSA, then washed and resuspended in 3 ml of TR* containing no CTC or BSA and their fluorescence monitored continuously while stirred at 37 in a fluorometer cuvette. Fluorescence was excited at 400 nm and emission read at 525 nm. Additions were: bethanechol, 100 uM; caerulein, 10 ng/ml; atropine, 5 uM; KCl, 58 mM.

We went so far as to measure the secretagogue-induced fluorescence change and the amylase released from the same sample of acini maintained in a cuvette (see Fig. 15). In this case, acini were stirred manually and the fluorescence measured pointwise. As observed previously, an increased loss of fluorescence occurred in the 5 minutes following bethanechol addition. At the same time, the rate of amylase release measured 5 to 15 minutes after the addition of bethanechol was increased by 240% over the control rate measured in the 15 minutes prior to adding bethanechol. This experiment indicated again that the CTC present in these acini did not interfere with normal stimulation of secretion.

To further study the relationship between amylase release and the decrease in CTC fluorescence produced by bethanechol we compared the dose-response relation for each of these actions. As shown in Fig. 16A, the ability of bethanechol to elicit a decrease in CTC fluorescence was dose dependent. Raising the bethanechol concentration increased the extent of fluorescence loss and decreased the response time. Figure 16B (open circles) shows that the fluorescence decrease in the first 8 minutes after bethanechol addition was half maximal at a bethanechol concentration of 20 uM and maximal at 100 uM and higher concentrations. Acini preincubated with CTC in an identical manner then incubated for 10 minutes (although in a separate experiment) released amylase in response to somewhat lower concentrations of bethanechol; half-maximal and maximal stimulation occurred at 5 uM and 30 uM bethanechol respectively. These doses are similar to those required to elicit amylase release from fragments of mouse pancreas (Williams, 1975a) and the maximal stimulation achieved in acini (200% to 400% above unstimulated values) is even greater than that usually obtained with fragments (150 to 250 above controls; see Williams, 1975a; Williams & Chandler, 1975). This is further evidence that CTC-loaded acini maintain an excellent secretory response. Although the dose response curves for stimulation-induced fluorescence changes and amylase release are different it is clear that bethanechol did elicit fluorescence changes at concentrations which stimulate

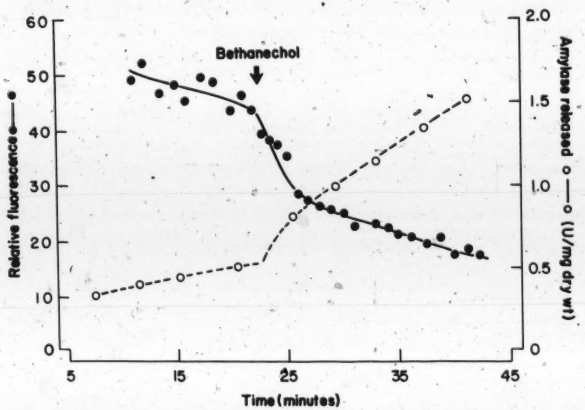
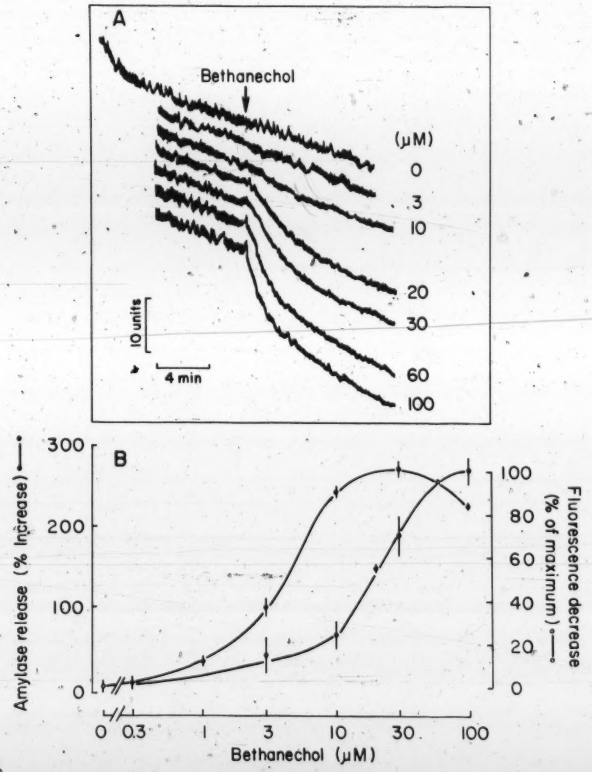


Fig. 15. CTC fluorescence of and amylase release from dissociated pancreatic acini. Acini were loaded with 100 uM CTC as described in Fig. 14, then stirred periodically in a fluorometer cuvette maintained at 37°. Fluorescence was monitored as in Fig. 14. Amylase released into the medium was measured by removing 0.2 ml aliquots of the suspension at the designated times, centrifuging at 8000 x g for 20 s, and assaying amylase in the supernatant; the cuvette contained 1.47 mg dry wt of acini per ml. Bethanechol (final conc. = 125 uM) was added at the arrow. These results are representative of 6 similar experiments.

Fig. 16. Dose dependence of the fluorescence decrease and amylase release elicited by bethanechol from acini loaded with CTC. Acini were preincubated 60 minutes in TR* containing 100 uM CTC and 1% BSA, then washed and resuspended in TR* containing no CTC or BSA.

A. Acini were placed in a fluorometer cuvette and their fluorescence monitored continuously as described in Fig. 14. Bethanechol, at the concentration indicated, was added at the arrow in each trace. All traces used cells from the same preparation. These results are representative of 4 similar experiments.

B. Acini (2 ml aliquots) were incubated in 25 ml flasks in a shaking water bath for 10 minutes. Amylase released during incubation (filled circles) is expressed as the relative increase above control values (i.e. % increase = (stimulated - control/control) x 100). Values are means ± S.E. of triplicate incubations in a single experiment. Results are representative of 3 similar experiments. Open circles indicate the relative decrease in fluorescence of CTC-loaded acini during the first 8 minutes of bethanechol stimulation. Values are means ± S.E. using data taken from traces like those in panel A recorded in 4 independent experiments. The intensity decrease observed after application of 100 uM bethanechol is designated 100% and that in control traces designated 0%.


A
Bethanechol
(μM)
0
3
10
20
30
60
100
10 units
4 min
B
Amylase release (% increase)
Fluorescence decrease (% of maximum)
300
200
100
0
100
80
60
40
20
0
0 0.3 1 3 10 30 100
Bethanechol (μM)

amylase release. However, the stimulation dependent phenomenon that these fluorescence changes monitor cannot be a direct readout of enzyme release since the doses of bethanechol that are half-maximal and maximal for the fluorescence change are 3 to 4 fold higher than those for amylase release and at supramaximal doses the fluorescence response continues to be maximal whereas amylase release is inhibited (see Fig. 16 and Williams, 1975a).

3. Divalent Cation Complexation of Chlorotetracycline Incorporated Into Acinar Cell Membranes

To demonstrate that the fluorescence of CTC incorporated into acinar cell membranes is dependent on the presence of divalent cations we compared the effect of extracellular Ca^{++} and Mg^{++} on the fluorescence of CTC in the presence of whole and sonicated acini. Fig. 17A shows that addition of acini to CTC in the presence of calcium resulted in a gradual increase in fluorescence almost reaching a plateau in 20 minutes. Addition of acini to CTC in the absence of divalent cations (EDTA, Fig. 17A) resulted in a fluorescence increase having a similar time course and a slightly lower magnitude. In contrast, if acini are broken by sonication to expose intracellular as well as plasma membrane to the extracellular medium and then added to CTC in EDTA -containing media almost no increase in fluorescence was observed (see Fig. 17B). The small increase seen was largely accounted for by light scattering of the added membranes. Upon addition of 1.33 mM Ca^{++} to these membranes, CTC fluorescence was rapidly increased 60 fold, reaching a plateau within 5 minutes (note difference in time scale between Fig. 17A and 17B). Complexing this calcium by adding 4.4 mM EDTA reduced the fluorescence intensity to the value observed prior to Ca^{++} addition. Addition of 1.33 mM Mg^{++} resulted in an intensity increase considerably smaller in magnitude (especially when considering that addition of Mg^{++} to CTC in the medium accounts for half of this increase) while a further addition of

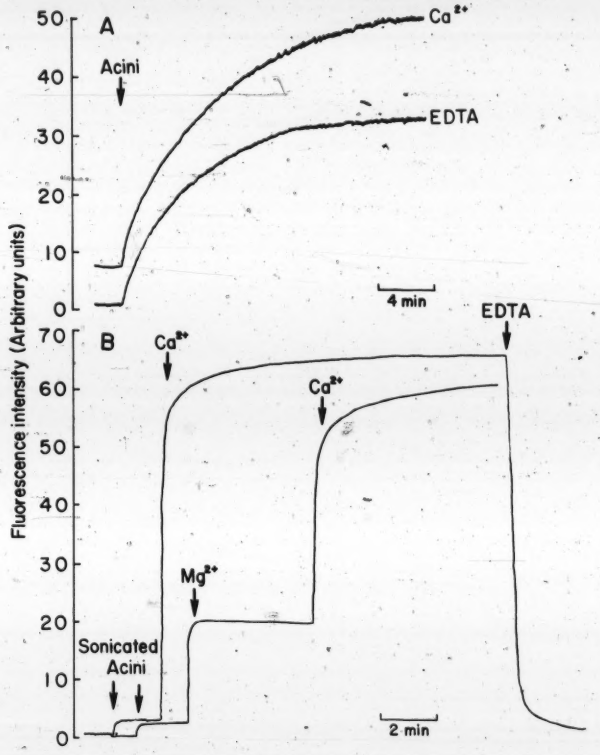


Fig. 17. Effect of extracellular divalent cations on CTC fluorescence from membranes of whole and sonicated acini.

A. Acini were washed and suspended in 0.15 ml of OCaOMgTR, then added (at the arrow) to a cuvette containing 3 ml of TR. The TR contained 100 uM CTC and either 1.28 mM calcium (but no magnesium) or 0.33 mM EDTA (with no added calcium or magnesium) as indicated.

B. Acini were sonicated in 0.15 ml of EDTA TR (see Methods) and added (at the heavy arrows) to 3 ml of OCaOMg TR containing 100 uM CTC. Additions were: Ca^{++}, 1.33 mM; Mg^{++}, 1.33 mM; EDTA, 4.4 mM.

In both A and B, fluorescence was excited at 400 nm and emission read at 525 nm; traces in both are representative of 4 similar experiments.

1.33 mM Ca^{++} increased fluorescence intensity almost to the value seen when Ca^{++} alone is present.

These results indicated that fluorescence of CTC in acinar cell membranes is markedly dependent on divalent cations as observed previously for other membrane systems (Caswell & Hutchison, 1971a). The greater fluorescence enhancement with Ca^{++} than Mg^{++} is similar to the selectivity seen for CTC incorporated into erythrocyte membranes (Hallett et al., 1971). The fluorescence increase seen when CTC is taken up by whole acini in the presence of EDTA is too large to be accounted for unless CTC is incorporated into intracellular membranes that are not exposed to EDTA and which bind divalent cations. The slower uptake of CTC by whole cells in either Ca^{++} or EDTA media was also consistent with CTC having to cross the plasma membrane to produce an enhancement in fluorescence.

Because the fluorescence spectra of the calcium and magnesium complexes of CTC differ, it was possible to determine which ion is complexed to CTC incorporated into membranes. Alcohol-water mixtures have been commonly used as a model system for studying the spectra of CTC's divalent cation complexes because spectra obtained in these solvents are similar to those observed for CTC in biological membranes (Caswell, 1972; Tajedal, 1975). Alcohols have the advantage that the ability of ions to form CTC complexes is not determined by the affinity and selectivity of the membrane binding site for calcium and magnesium but rather by the properties of CTC itself. In 90% methanol - 10% 10 mM Tris HCl, pH 7.4, in 130 mM NaCl the excitation and emission spectra of Ca^{++}-CTC and Mg^{++}-CTC and uncomplexed CTC all differ substantially as shown in Fig. 18 (solid symbols). These spectra have been normalized to more clearly demonstrate these differences; the peak intensities for CTC in the presence of 1 mM calcium and 1 mM magnesium are 45 and 66 fold greater, respectively, than the intensity of CTC in the presence of EDTA. This demonstrates that CTC fluorescence is very sensitive to divalent cation complexation in this solvent. Excitation and emission maxima for free CTC (filled circles) are at 382 nm and 536 nm (emission spectrum not

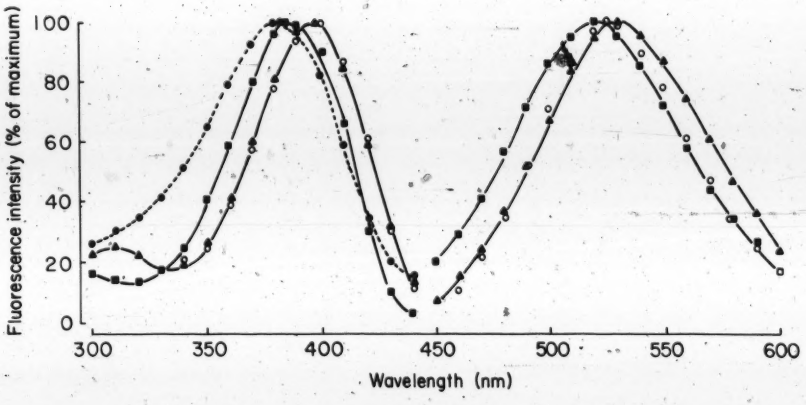


Fig. 18. Excitation and emission spectra of CTC and its divalent cation complexes in methanol-Tris Ringer and comparison of these to spectra from unstimulated acini loaded with CTC. To 10 uM CTC in 90% methanol--10% 10 mM Tris HCl, pH 7.4, in 120 mM NaCl was added 1 mM Ca^{++} (▲—▲), 1 mM Mg^{++} (■—■), or 0.33 mM EDTA (●--●), and excitation and emission spectra recorded at 30°. Unstimulated acini loaded with CTC (○) were suspended in TR* and stirred continuously at 37° in a cuvette while spectra were recorded. Relative peak intensities for excitation spectra of CTC in the presence of Mg^{++}, Ca^{++}, EDTA, and acini were 27.6, 18.8, 0.42, and 28.5 respectively. In all fluorescence was excited at 400 nm and emission read at 525 nm.

shown) while those for Mg^{++}-CTC are at 385 nm and 519 nm and for Ca^{++}-CTC at 398 nm and 529 nm. Thus, the entire excitation and emission peaks for Mg^{++}-CTC are each shifted 10 nm to the blue compared to these peaks for Ca^{++}-CTC. Spectra for pancreatic acini preincubated in CTC and then washed free of extracellular probe are shown for comparison (open symbols). It is clear that the excitation spectrum of acinar cell-associated CTC (λ max. = 400 nm) is identical to that of the Ca^{++} complex of CTC in 90% methanol. It cannot be accounted for by the spectrum of uncomplexed or the Mg^{++} complexed probe in 90% methanol, or by the excitation spectra of either divalent cation complex in aqueous medium (λ max. = 385 and 383 nm, respectively, in Tris Ringer). The emission spectrum of cell-associated CTC is not as conclusive since it appears to be a narrower peak that overlaps in part the spectrum of each complex. Yet, the spectrum is clearly not that of uncomplexed CTC ($\lambda_{max.}$ = 536 nm, not shown) and its maximum at 526 nm suggests that it is CTC complexed to a mixture of Mg^{++} and Ca^{++}, mostly the latter. From these spectra we conclude that fluorescence of CTC in acinar cells is emitted from probe molecules complexed to divalent cations, primarily Ca^{++}, in a membrane environment. This conclusion is corroborated by fluorescence microscopy which shows that the distribution of CTC fluorescence in acinar cells is similar to that of intracellular membrane (see Fig. 23).

4. Preferential Loss of Fluorescence From the Calcium Complex of CTC during Secretory Stimulation

The spectral differences between the Ca^{++} and Mg^{++} complexes were further utilized in studying changes in divalent cation complexation to CTC in pancreatic acini during stimulation of secretion. As shown in the inset of Fig. 19, application of caerulein to CTC-loaded acini elicited a rapid intensity decrease. Spectra (Fig. 19) taken 2 minutes before stimulation were similar to those described above for CTC in unstimulated acini and had excitation and

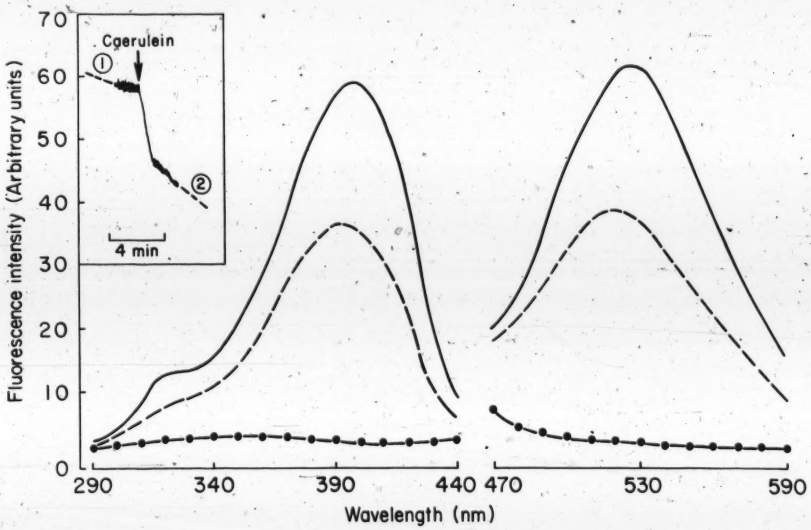


Fig. 19. Excitation and emission spectra of CTC-loaded acini before and after stimulation with caerulein. Acini were loaded with CTC as described in Fig. 14, then washed and resuspended in 3 ml of TR* containing no CTC or BSA and their fluorescence monitored while being stirred at 37° in a cuvette. Solid line spectra were taken 2 minutes before stimulation at 1 in the inset and dashed spectra taken 4 minutes after stimulation at 2 in the inset. Caerulein, 17 ng/ml, was added at the arrow. Light scattering of acini containing no CTC (●—●—●) is also shown. Fluorescence was excited at 400 nm and emission read at 525 nm.

emission maxima at 398 nm and 527 nm respectively. Five minutes after stimulation the peak fluorescence intensity had decreased 39% and excitation and emission maxima had shifted to 393 nm and 521 nm respectively. In control experiments the spectra of CTC in acini not stimulated but stirred continuously in a cuvette for 40 minutes underwent no shifts although they did sustain a 57% loss in intensity. This suggests that stimulation may induce loss of signal from a specific subcellular compartment rather than simply facilitating outflux by the normal routes.

If these spectra are normalized, as shown in Fig. 20A, it can be seen that the entire excitation and emission peaks from unstimulated cells (open circles) are shifted 6 nm to shorter wavelengths after stimulation (filled circles). Cholinergic stimulation shifted these peaks in an identical manner. As shown by the normalized spectra in Fig. 21, unstimulated acini had excitation and emission maxima at 399 nm and 526 nm respectively while 9 minutes after simulation with bethanechol, these maxima were shifted to 393 nm and 520 nm respectively.

Judging from the model studies in 90% methanol that were described above, the fluorescence before stimulation is largely from calcium complexed CTC while that after stimulation is largely from the magnesium complexed probe. This conclusion is further substantiated by the experiment illustrated in Fig. 20B. Spectra of identical aliquots of CTC-loaded acini were resuspended in medium containing either Ca^{++} or Mg^{++} (but not CTC). The cells were then broken by sonication to expose CTC-laden intracellular membranes to the divalent cation contained in the extracellular medium and the spectrum recorded immediately. As shown in Fig. 20B, CTC incorporated into acinar cell membranes and exposed to Ca^{++} (filled triangles) exhibited excitation and emission maxima at 399 nm and 325 nm respectively--almost identical to the maxima observed in unstimulated whole acini (open circles). In contrast the excitation and emission peaks for membrane incorporated CTC incorporated into acini membranes and exposed to Mg^{++} were shifted about 7 nm and exhibited maxima at 393 nm and 520 nm

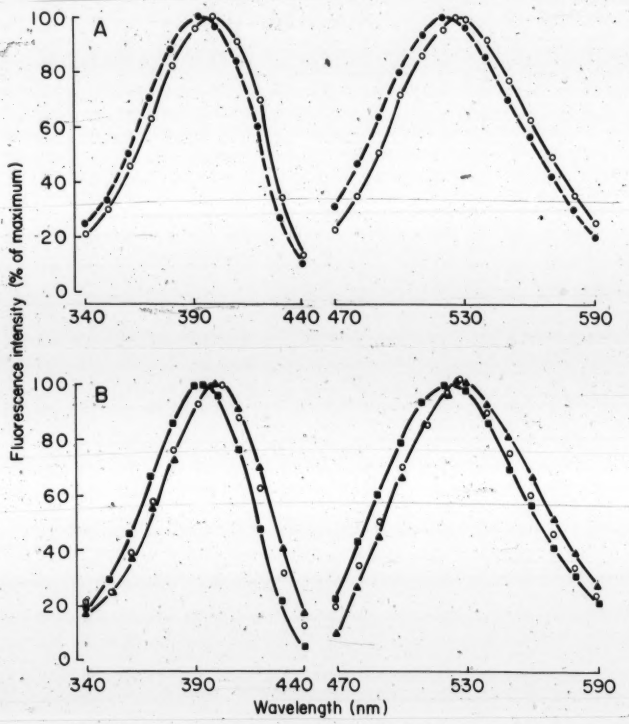


Fig. 20 A. Excitation and emission spectra of acini before (○) and after (●) stimulation with caerulein. Spectra from Fig. 19 were corrected by subtracting the light scattering of acini not incubated with CTC, then normalized to clearly demonstrate the spectral shifts produced by caerulein stimulation. These spectral shifts were observed in each of 4 experiments.

Fig. 20 B. Excitation and emission spectra of CTC in membranes of acini broken by sonication and exposed to calcium or magnesium. Acini were loaded with CTC as described in Fig. 14, then washed, resuspended in TR containing 1.33 mM Ca^{++} (▲) or Mg^{++} (■) (but not both) and sonicated as described in Methods. The sonicated suspension was placed in a cuvette stirred at 37° and spectra recorded immediately. Fluorescence was excited at 400 nm and emission read at 525 nm. Spectra were corrected for light scattering then normalized. Spectra of acini loaded with CTC and suspended in TR* (○) are shown for comparison. Relative peak intensities for excitation spectra were 19.7 (▲), 17.6 (■), and 27.9 (○).

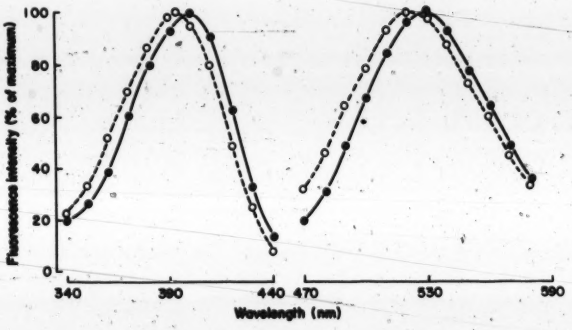


Fig. 21. Excitation and emission spectra of CTC-loaded acini 2 minutes before (●) and 9 minutes after (○) bethanechol stimulation. Pancreatic acini were loaded with CTC and their fluorescence monitored as described in Fig. 14. Spectra were corrected by subtracting the light scattering of an acini suspension not exposed to CTC, then normalized to demonstrate the spectral shifts. Fluorescence intensity of maxima before and after stimulation with 100 uM bethanechol was 70 and 45 units respectively. These shifts were observed in each of 5 experiments.

respectively--very close to those maxima observed for CTC in acini after stimulation. We concluded from these data that there is a preferential loss of signal from Ca^{++} - complexed CTC during stimulation.

5. Control Experiments With Divalent Cation-Insensitive Probes

Further evidence that the decrease in CTC fluorescence during stimulation resulted from dissociation of CTC's divalent cation complexes came from experiments on acini loaded with oxytetracycline (OTC) or 1-anilino-8-naphthalene sulfonate (ANS). These probes are similar to CTC in that they undergo fluorescence enhancement when transferred from aqueous to membrane environments but unlike CTC in that this enhancement is relatively insensitive to the amount of Ca^{++} and Mg^{++} bound to the membrane. Fig. 22 (upper panel) shows that acini preincubated with 200 uM ANS (in the absence of albumin since BSA inhibited its uptake) displayed a gradual decline of fluorescence which was entirely unaffected by the addition of bethanechol to stimulate secretion. Acini preincubated with 100 uM CTC (in the absence of BSA) showed the usual intensity decrease during stimulation (Fig. 22, upper panel). Likewise the fluorescence of acini loaded with 100 uM OTC (shown in Fig. 22, lower panel) was unaffected by bethanechol addition. In contrast, acini preincubated with 20 uM CTC to obtain a fluorescence intensity equivalent to that of OTC-loaded acini (but 25% lower than that obtained with 100 uM CTC) exhibited a clear decrease of fluorescence upon stimulation. Preincubation of acini with OTC or ANS had no effect on their ability to secrete amylase under basal or stimulated conditions, as shown in Table 3.

One possibility was that the stimulated decrease of CTC fluorescence was the result of CTC partitioning out of intracellular membranes after dissociation of its divalent cation complexes. Studies with isolated mitochondria have indicated that both processes

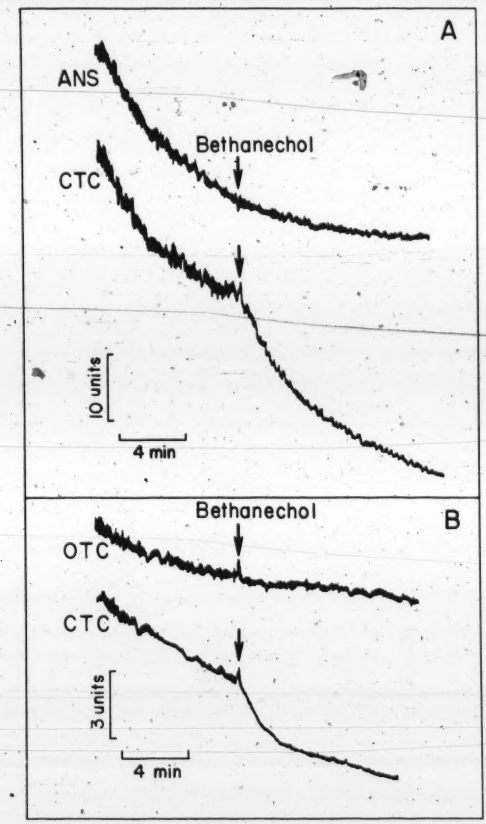


Fig. 22. Fluorescence of acini loaded with 1-anilino-8-naphthalenesulfonate (ANS) or oxytetracycline (OTC) is not affected by bethanechol stimulation. In the upper panel, acini were preincubated 60 minutes with 200 uM ANS or 100 uM CTC in TR* containing no BSA; in the lower panel acini were preincubated 60 minutes with 100 uM OTC or 20 uM CTC in TR* containing 1% BSA. After preincubation, acini were washed and resuspended in TR* containing no BSA or fluorescence probes, and stirred continuously at 37° in a fluorometer cuvette. Fluorescence was excited and emission read at 380 nm and 470 nm, respectively, for ANS, at 380 nm and 510 nm for OTC, and at 400 nm and 525 nm for CTC. Bethanechol, 100 uM, was added at the arrows.

TABLE 3

AMYLASE AND FLUORESCENT PROBE RELEASE FROM PANCREATIC ACINI PREINCUBATED WITH CHLOROTETRACYCLINE (CTC), 1-ANILINO-8-NAPHTHALENESULFONATE (ANS), OR OXYTETRACYCLINE (OTC)

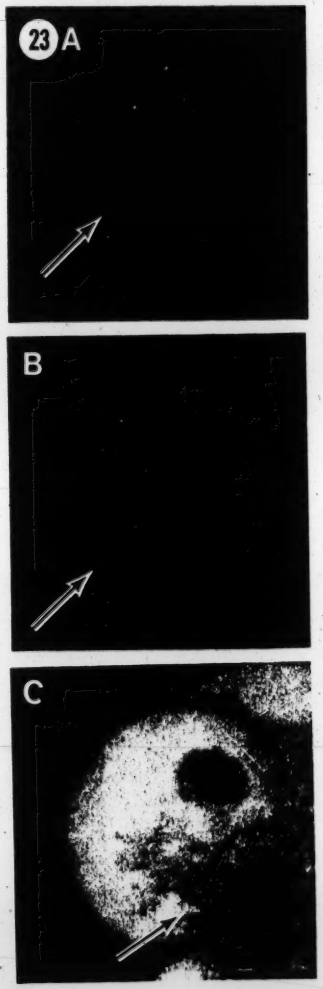
Probe in Preincubation Medium		Amylase Release (U/10 min/mg protein)		Fluorescent Probe Release (nmoles/10 min/mg protein)	
		Control	Bethanechol (100 uM)	Control	Bethanechol (100 uM)
Exp. 1	CTC	0.32 ± 0.05	1.03 ± 0.04	0.55 ± 0.03	1.17 ± 0.07
Exp. 2	ANS	0.51 ± 0.06	1.58 ± 0.25	6.70 ± 0.50	6.70 ± 0.50
	OTC	0.49 ± 0.06	1.43 ± 0.06	0.12 ± 0.01	0.14 ± 0.02
	None	0.47 ± 0.03	1.24 ± 0.12	--------	--------

Acini were preincubated 60 minutes in TR* containing either 100 uM CTC and 1% BSA, or 100 uM OTC and 1% BSA, or 200 uM ANS and no BSA, then washed and resuspended in TR* containing no BSA or fluorescent probes. Two ml aliquots of this suspension were incubated in 25 ml flasks for 10 minutes at 37° and amylase and fluorescent probe released into the incubation medium assayed. Values are means ± S.E. of triplicate or quadruplicate incubations. The results are representative of five experiments like No. 1 and two sets of experiments like No. 2.

occur when divalent cations dissociate from membranes containing CTC. Consistent with this possibility is that release of CTC into the extracellular medium was increased 113% during the first 10 minutes of stimulation (see Table 3). In contrast, stimulation did not effect the outflux of ANS or OTC--probes which also displayed no intensity changes during stimulation. We conclude then that the decrease in CTC fluorescence and its increased release into the medium during stimulation both stem from its affinity for divalent cations in acinar cell membranes since the fluorescence and efflux of ANS and OTC, probes that interact with Ca^{++} and Mg^{++} only weakly, are unaffected.

The absence of any change in ANS or OTC fluorescence during stimulation also gave assurance that those changes seen for CTC fluorescence did not result from optical effects. It is conceivable that the changes in cell structure (e.g. granule movement or exocytosis) or in cell grouping (e.g. groups of cells might form or disperse) during stimulation, or the production of some chemical either absorbing incident or emitted light could produce changes in CTC fluorescence. This seems unlikely because optically ANS and OTC are very similar to CTC. The excitation and emission maxima of OTC (380 nm and 510 nm, respectively) are nearly identical to those of CTC (400 nm and 520 nm, respectively). In addition, ANS, OTC, and CTC all display a similar pattern of emission from isolated acinar cells (see Fig. 23). The nuclei are nonfluorescent and areas containing zymogen granules (arrows) have a lower fluorescence intensity. This pattern (as discussed later) is similar to the distribution of intracellular membranes and reflects the fluorescence enhancement that these probes display in a membrane environment. Thus if fluorescence had been altered optically during stimulation, acini loaded with ANS and OTC should have displayed similar fluorescence changes.

Fig. 23. Fluorescence images of isolated pancreatic acinar cells incubated in TR* containing 200 uM ANS, 100 uM OTC, or 100 uM CTC. Arrows indicate areas containing zymogen granules that fluoresce with a lower intensity. x 1170.



6. Detection of an Intracellular Compartment Whose Interaction With Chlorotetracycline Is Stimulation Dependent

To determine whether the reduction in CTC fluorescence during stimulation is paralleled by a loss of CTC from acini, we measured the CTC content of acini and of the incubation medium during application of bethanechol. Fig. 24A (open circles) shows that acini which were preincubated in 100 uM CTC for 60 minutes, then washed and resuspended in medium without CTC, contained about 6.5 nanomoles of CTC per mg protein. This content decreased slowly (about 1 nanomole per mg protein over the next 40 minutes) and could be completely accounted for by CTC appearing in the medium (Fig. 24B, open squares). Loss of CTC during stimulation (Fig. 24A, filled circles) was much greater--33% in the first 20 minutes of stimulation compared to only a 10% loss from control acini during the same period. Again this stimulated decrease of CTC content was reasonably well accounted for by increased amounts of CTC appearing in the incubation medium (Fig. 24B, filled squares). Thus, increased release of CTC and of amylase (see Fig. 24C) was maintained throughout the first 20 minutes of bethanechol stimulation. For comparison, we monitored the fluorescence intensity of CTC-loaded acini under similar mild conditions (incubated in a cuvette stirred manually rather than continuously with a magnetic stirrer) and found that the fluorescence intensity loss upon stimulation was much more rapid than the reduction of CTC content (see Fig. 24D). Of the intensity lost in the first 20 minutes, 67 $\pm$ 0.7% (mean $\pm$ S. E., n = 12) came in the first five minutes of stimulation. This indicates that the decrease of fluorescence intensity precedes the chemical loss of CTC from the acini. These results suggested that the stimulated loss of CTC fluorescence is accompanied or followed by partitioning of CTC out of some set of intracellular membranes and that probe molecules released intracellularly must diffuse across the plasma membrane before being "chemically" lost from the cell.

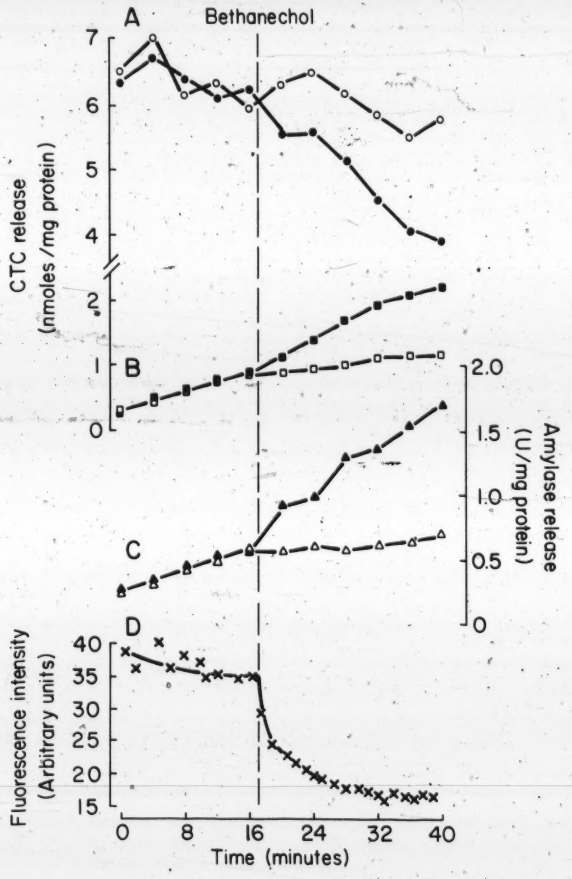


Fig. 24. CTC content, CTC release, amylase release, and fluorescence of pancreatic acini during stimulation with bethanechol. Acini were preincubated for 60 minutes in TR* containing 100 uM CTC and 1% BSA, then washed and resuspended in TR* containing no CTC or BSA. In experiments which determined CTC content of acini (trace A), CTC released into the medium (trace B), and amylase released into the medium (trace C), 7 ml aliquots of the acini suspension were incubated in a 25 ml flasks at 37 with (filled symbols) or without (open symbols) addition of bethanechol (100 uM) at 16 minutes (arrows). At the times designated, 0.5 ml aliquots were removed, centrifuged, and the supernatant assayed for CTC and amylase. Pellets were resuspended in 1 ml of TR*, then sonicated and assayed for CTC and protein as described in Methods. In D, CTC-loaded acini were incubated in a cuvette at 37 , periodically stirred and fluorescence measured at the times indicated. Fluorescence was excited at 400 nm and emission read at 525 nm; bethanechol, 100 uM, was added at the arrow.

Studies of chlorotetracycline uptake also suggested that secretory stimulation alters the ability of some set of intracellular membranes to interact with the probe. We measured the CTC content of acini during incubation in 100 uM CTC and found that bethanechol stimulation did not affect CTC uptake during the first 20 minutes (see Fig. 25A). After this time uptake was markedly reduced in stimulated acini (filled triangles). The lack of an effect at early times indicated that stimulation did not affect plasma membrane permeability to the probe. The reduced probe content of stimulated acini at long times suggested that some cell compartment that usually takes up CTC slowly is refractory to uptake.

This time course for CTC uptake in stimulated and unstimulated acini corresponds well with that for fluorescence intensity changes observed during CTC uptake (see Fig. 25B); at times less than 15 minutes there is little difference in the fluorescence of stimulated and unstimulated acini while at longer times stimulated acini have a significantly lower intensity. This provides another instance in which changes in acini fluorescence are accompanied by corresponding changes in CTC content. At long times, however, there does seem to be an accumulation of CTC that does not contribute to the fluorescence.

Of course, we prefer to think that the results of Fig. 25 mean that CTC does not reach (and therefore does not probe) the stimulation-sensitive compartment at early times. But it is also possible that CTC reaches this compartment quickly but that the compartment is altered only after 15 minutes of stimulation. To differentiate between the possibilities we applied bethanechol to acini at different stages of CTC uptake and recorded the acute fluorescence changes. As shown in Fig. 26, application of bethanechol after 10 minutes of CTC uptake had almost no effect while application at 22 minutes or later times produced immediate decreases in fluorescence of increasing magnitude at successively later times up to 62 minutes. This indicates quite clearly that CTC fluorescence probes a stimulation-induced alteration of an intracellular compartment that

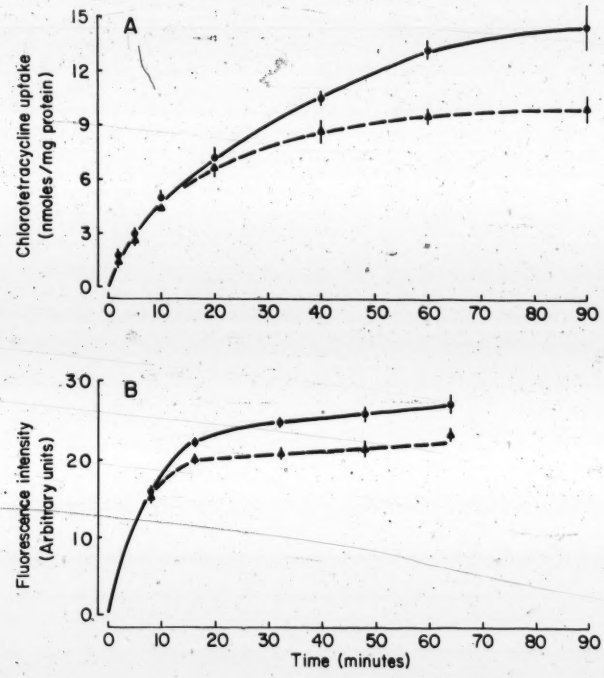


Fig. 25. Uptake of CTC by pancreatic acini in the presence (▲) or absence (●) of bethanechol.

A. Acini were washed in OCaOMg TR then at time zero resuspended in 5 to 7 ml of TR containing 100 uM CTC and 1.28 mM Ca^{++} (but no Mg^{++} or BSA). Bethanechol, 100 uM, was present as indicated. At designated times, 0.7 ml aliquots were withdrawn, acini separated by suction filtration and assayed for CTC and protein as described in Methods. Values are means ± S.E. of 4 to 8 determinations on 4 cell preparations.

B. Acini were washed in OCaOMg TR and resuspended in TR containing 100 uM CTC and 1.28 mM Ca^{++} (but no Mg^{++} or BSA). Bethanechol was present as indicated. Three ml aliquots of this acini suspension were incubated at 37° in 25 ml flasks and at the designated times poured into a cuvette and fluorescence read. Fluorescence was excited at 400 nm and emission read at 525 nm. Values are means ± S.E. of triplicate incubations. The results are representative of 2 similar experiments.

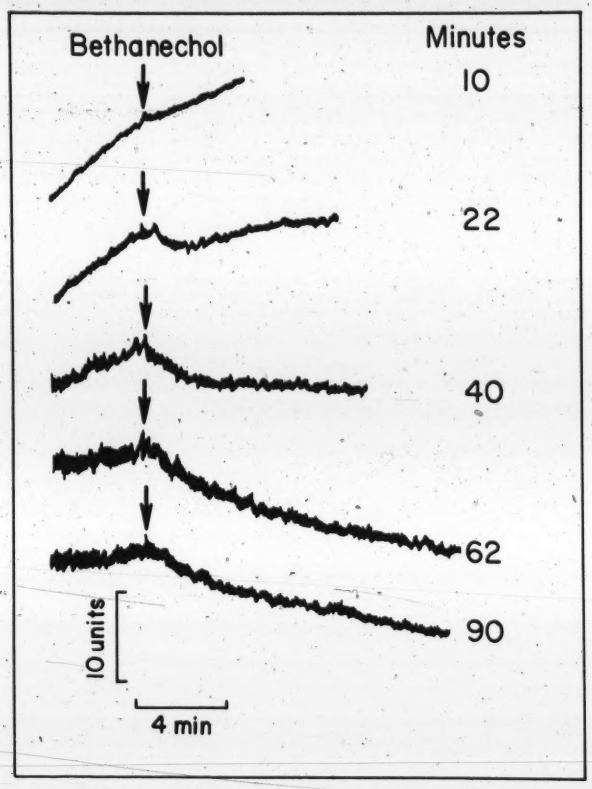


Fig. 26. Effect of bethanechol on the fluorescence of pancreatic acini during CTC uptake. Acini were washed in OCaOMg TR and at time zero resuspended in TR containing 100 uM CTC and 1.28 mM Ca^{++} (but no Mg^{++} or BSA). Three ml aliquots of the acini suspension were incubated in 25 ml flasks and at 5 minutes before bethanechol addition were poured into a fluorometer cuvette, stirred at 37° and their fluorescence monitored continuously. Bethanechol, 100 uM, was added to each sample at the arrow after exposure to CTC for the length of time indicated. Excitation was at 400 nm and emission read at 525 nm. These results are representative of 4 similar experiments.

occurs quite rapidly but that CTC reaches this compartment only after 15 to 20 minutes of uptake.

7. Effect of Metabolic Inhibitors on CTC Fluorescence From Dissociated Acini

Because CTC is known to accumulate in mitochondria of living cells (DuBuy & Showacre, 1961) and is able to monitor divalent cation accumulation and release from isolated mitochondria (Caswell & Hutchison, 1971a; Caswell, 1972; Luthra & Olsen, 1976) we looked for a mitochondrial component of the CTC signal from acini by using specific mitochondrial inhibitors. As can be seen in Fig. 27 (upper trace) application of m-chlorophenyl carbonyl cyanide (ClCCP), an uncoupler of oxidative phosphorylation, to CTC-loaded acini resulted in a rapid loss of CTC fluorescence. Fluorescence was decreased abruptly by a small amount, then continued to decrease for about 10 minutes at a rate much faster than that observed prior to ClCCP addition. In contrast, if the acini were stimulated with bethanechol and the usual fluorescence changes allowed to run their course, addition of ClCCP had no effect except for the small abrupt intensity decrease. (This abrupt decrease is thought to be artifactual since ClCCP in the same concentration range reduces fluorescence of CTC incorporated into erythrocyte membranes by 10%. Since this effect is proportional to ClCCP concentration and ClCCP exhibits an absorption maximum at 380 nm it is probably due to the inhibitor absorbing incident light). Similar results were obtained using the mitochondrial electron transport inhibitors rotenone, antimycin A, and NaCN. Experiments using the latter two are depicted in Fig. 27B and 27C. Addition of either inhibitor to CTC-loaded acini produced a rapid loss of fluorescence; but again, if acini were first stimulated with bethanechol these inhibitors were without effect. This strongly suggested that bethanechol and mitochondrial inhibitors decrease CTC fluorescence at the same intracellular site.

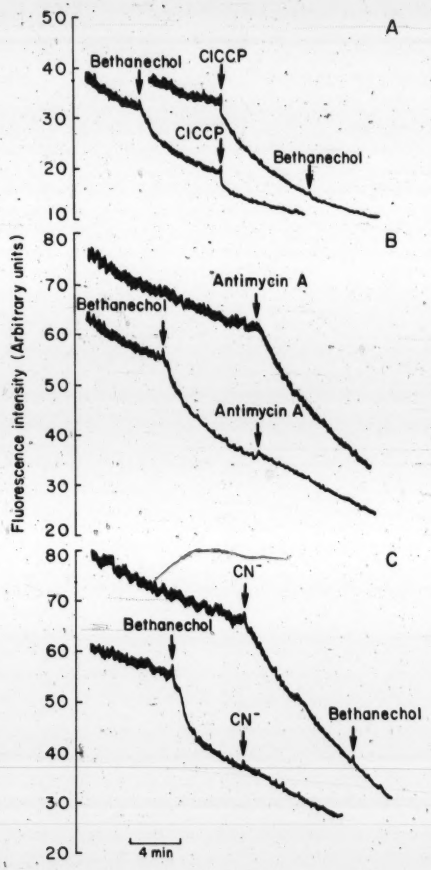


Fig. 27. Effect of metabolic inhibitors on fluorescence of pancreatic acini loaded with CTC. Acini were preincubated 60 minutes in TR* containing 100 uM CTC and 1% BSA, then washed and resuspended in 3 ml of TR* containing no CTC or BSA. The suspension was placed in a fluorometer cuvette, stirred at 37° and fluorescence monitored continuously. Excitation was at 400 nm and emission read at 525 nm. Additions were: bethanechol, 100 uM; ClCCP, 6.7 uM; antimycin A, 6.7 uM; NaCN, 1 mM. Traces in each panel are representative of 3 to 5 similar experiments.

Based on the evidence given above, we can presume that they all do so by causing release of calcium from that site. The site could be mitochondria themselves since metabolic inhibitors are known to release mitochondrial calcium, or it could be some other compartment that requires mitochondrial ATP to hold on to its calcium store.

8. Effect of Extracellular Divalent Cations on CTC Fluorescence From Dissociated Acini

A portion of the fluorescence from CTC-loaded acini was found to be sensitive to changes in divalent cations. Adding 4.4 mM EDTA to acini incubated in TR* caused a rapid drop in their fluorescence to 80-85% of the initial value within two minutes (third trace, Fig. 28A); thereafter their fluorescence continued to drop at a rate faster than usual. In contrast, adding EDTA to acini incubated in TR* that had no Ca^{++} or Mg^{++} to start with did not effect the rate at which they lost fluorescence (first traces, Fig. 28A and B). Presumably this means that there is a pool of CTC that probes superficial divalent cations, which can be depleted quickly by simply removing divalent cations from the extracellular medium. But more than this, since CTC fluorescence continues to drop at an increased rate even 5 to 10 minutes after EDTA addition, it would appear that chelation of extracellular divalent cations may also deplete calcium and magnesium from intracellular sites that are more slowly exchangable.

Perhaps the most important experiment is the following: when either bethanechol (Fig. 28A) or caerulein (Fig. 28B) is added to CTC-loaded acini in media lacking calcium and magnesium (regardless of whether EDTA was present or absent) a rapid loss of fluorescence is observed very much similar to what is seen normally when extracellular divalent cations are present. It is unlikely that this is displacement of some superficial pool of cations. Nearly all cells in this acini preparation are directly exposed to the bulk medium and in

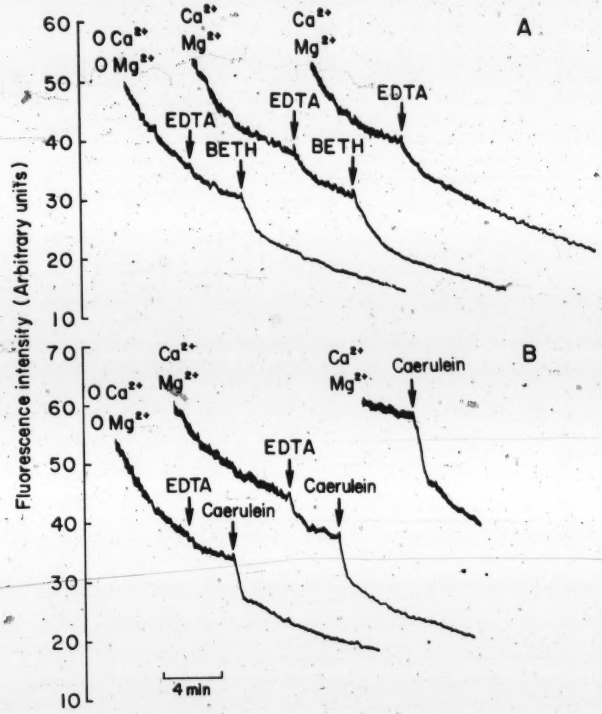


Fig. 28. Effect of extracellular divalent cations on the fluorescence of CTC-loaded pancreatic acini and on fluorescence changes produced by secretagogues. Acini were loaded with CTC and their fluorescence monitored as described in Fig. 27. The resuspension medium was 3 ml of TR containing 1.28 mM Ca^{++} and 0.56 mM Mg^{++} when indicated at the beginning of each trace. Additions were: EDTA, 4.4 mM; bethanechol, 100 uM; caerulein, 17 ng/ml. Each trace is representative of 2 to 4 similar experiments.

these experiments (first traces, Fig. 28A and 28B) the medium had lacked calcium and magnesium for 15 minutes prior to stimulation. However, the fluorescence loss produced by either caerulein or bethanechol in the absence of divalent cations is somewhat smaller than that seen when extracellular divalent cations are present (in the case of caerulein, this may be seen if the first and third traces of Fig. 28B are compared). I perfer to think that this means the CTC-probed pool of calcium inside cells is slightly depleted beforehand by the incubation in calcium-free medium.

9. Effects of Ionophore A23187 on CTC Fluorescence From Dissociated Acini

Application of 1 uM A23187 to acini loaded with CTC and suspended in TR* (Ca^{++} = 1.28 mM, Mg^{++} = 0.56 mM) resulted in an obvious decrease in fluorescence after a lag which ranged from 1 to 4 minutes in five experiments (see Fig. 29). This concentration of ionophore stimulated amylase release but did not damage the cells (see Fig. 12). Furthermore, if acini were incubated in TR* and 4.4 mM EDTA added to chelate extracellular calcium and magnesium (middle trace, Fig. 29), addition of A23187 resulted in an extremely rapid and extensive loss of CTC fluorescence; this action occured with no lag. This indicates that A23187 releases divalent cations from an intracellular compartment and suggests that the ionophore may trigger amylase release by releasing intracellular stores of calcium in addition to increasing influx of extracellular calcium. Possibly, the action of A23187 is more rapid in the absence of extracellular calcium and magnesium because it is taken up extremely rapidly under these conditions (Fig. 3A and 3B).

In contrast, addition of A23187 to acini in calcium-free medium (with magnesium still present) produced no fluorescence changes. It is not clear in this case why the ionophore failed to release divalent cations but it does correlate with the observation

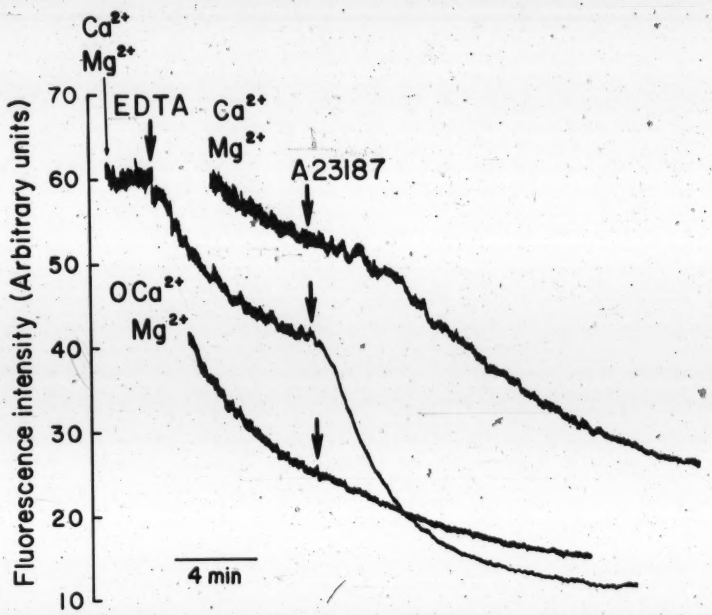


Fig. 29. Effects of the ionophore A23187 on fluorescence of CTC-loaded pancreatic acini. Acini were loaded with CTC and their fluorescence monitored as described in Fig. 27. The resuspension medium was 3 ml of TR containing 1.28 mM Ca^{++} and/or 0.56 mM Mg^{++} as indicated at the beginning of each trace. Additions were: A23187, 1 uM; EDTA, 4.4 mM. The results are representative of 3 similar experiments.

that even high concentrations of the ionophore did not release amylase under these conditions (Fig. 8).

10. Subcellular Localization of Chlorotetracycline By Fluorescence Microscopy

The usefulness of chlorotetracycline as a probe for divalent cations is considerably increased by the fact that it can be localized by fluorescence microscopy. Isolated acinar cells proved to be a favorable preparation for this purpose because the subcellular distribution of fluorescence in single cells could be observed. Localization of CTC in the pancreatic acinar cell was complicated by the variety of organelles and the high density of membranes in this cell, as illustrated in the electron micrograph of Fig. 30. Isolated cells, although they were spherical, retained much of the regional organization that one sees in the pyramidal-shaped cell of intact pancreas. One or occasionally two nuclei were eccentrically placed, zymogen granules were confined to well-defined clusters usually abutting on the plasma membrane, and rough endoplasmic reticulum filled nongranular regions. Golgi membranes, characteristically found between nucleus and zymogen granules in intact tissues, appeared to be distributed throughout the zymogen granule cluster in isolated cells. Fig. 30 also demonstrates that acinar cells, fixed after incubation in 0.1 or 0.5 mM CTC for 100 minutes and examined by electron microscopy, displayed completely normal ultrastructure in four independent experiments.

When visualized by fluorescence microscopy, CTC emission from acinar cells appeared yellow-green at low intensities and yellow at higher intensities. After 15 to 30 minutes of incubation in 0.1 mM CTC (no BSA present) acinar cells varied in intensity but by 60 minutes the large majority of cells exhibited an intense yellow fluorescence. When cells were first placed on the slide they were rounded and exhibited a uniform emission as shown in Fig. 31. Two

Fig. 30. Ultrastructure of an isolated mouse pancreatic acinar cell incubated for 100 minutes in TR containing 500 uM CTC. Cell components are similar to those of intact tissue consisting of abundant zymogen granules, rough endoplasmic reticulum, and mitochondria. The region containing zymogen granules is well defined and interlaced with golgi vesicles and condensing vacuoles. x 7100

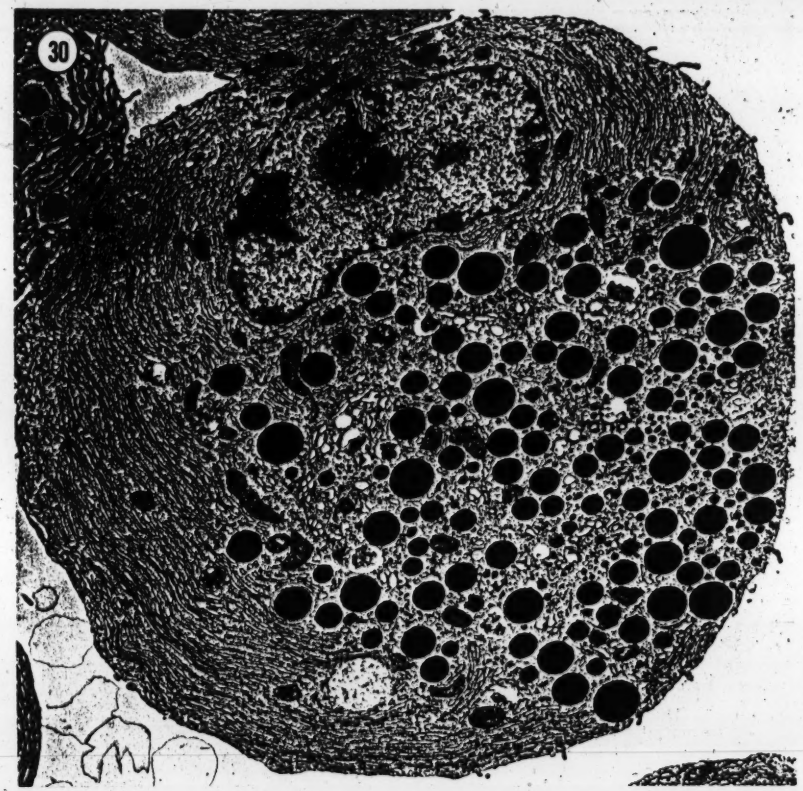

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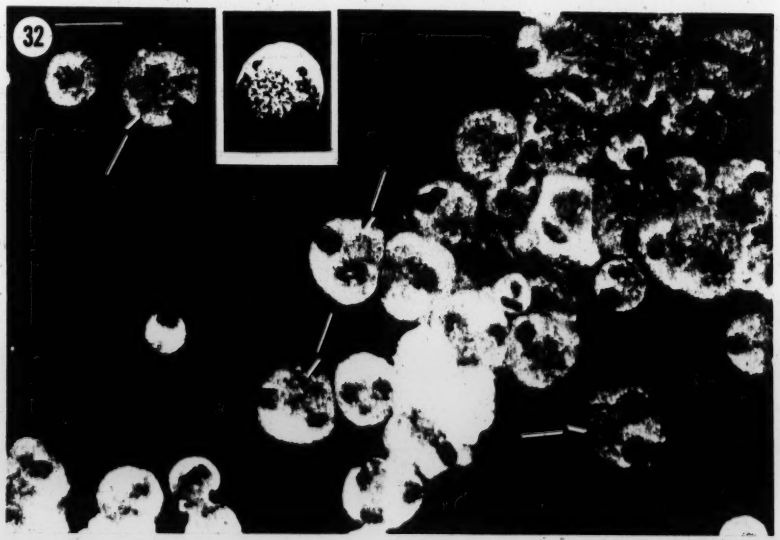
Fig. 31. Fluorescence of isolated mouse pancreatic acinar cells incubated in TR containing 100 uM CTC. Nuclei (N) are nonfluorescent; zymogen granule cluster s (Z) have a lower intensity. x 950.

Fig. 32. Fluorescence of isolated pancreatic acinar cells incubated in TR containing 100 uM CTC after cells have adhered to the glass slide and flattened out. Zymogen granule clusters are indicated by arrows. The inset, a dark field image of the adjacent cell in the main figure, demonstrates how granule containing areas were identified. x 640.


31
N
Z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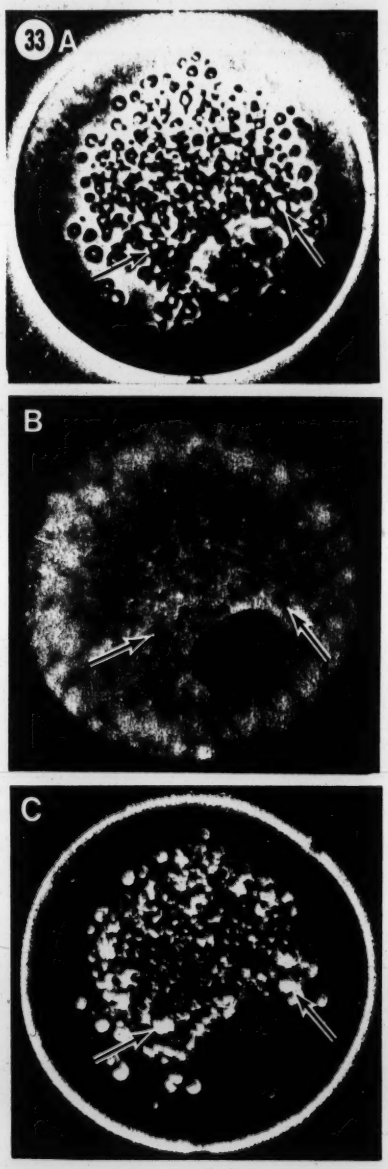
exceptions were noted. Nuclei (N) appeared as "black holes" while areas containing zymogen granules (identified by intense scattering of incident light when the barrier filter was changed from a 500 nm cutoff to a 470-650 nm bandpass filter) exhibited fluorescence of lower intensity.

After several minutes, at which time the cells had adhered to the slide and flattened out, a much clearer picture of the fluorescence distribution could be obtained. As shown in Fig. 32, the areas containing zymogen granules (arrows) were clearly demarcated by their lower intensity (the inset shows how these areas were identified) and the nuclei, as in Fig. 31, were nonfluorescent. Other areas of these cells did contain a uniform fluorescence although superimposed on this was a punctate pattern of emission that had not been apparent when the cells were rounded. (Note the stippled appearance of many cells in Fig. 32). If cells were incubated with CTC in TR* containing 1% BSA this punctate pattern of emission was apparent in a majority of the cells; even under the best of conditions, however, this pattern required careful observation because of the intense uniform fluorescence also present. In some cells it could not be seen at all. In addition, emission from the punctate pattern faded quickly. Fading is characteristic of CTC emission from mitochondria in other cells (DuBuy & Showacre, 1961; Allison & Young, 1964).

The uniform pattern of fluorescence is most clearly shown in Fig. 33 depicting an isolated cell that was compressed between the slide and cover glass following partial evaporation of the incubation medium. The phase contrast image (Fig. 33A) identifies the nucleus and zymogen granules. When compared with the fluorescence image (Fig. 33B) it confirms the observation that the nuclei do not fluoresce and that areas containing granules are of lower intensity. In favorable photographs such as this, the position of individual zymogen granules (arrows in Fig. 33A) could be matched to nonfluorescent "shadows" (arrows in Fig. 33B). Fig. 33C showing incident light scattered from individual granules, not only confirms the position of previously

Fig. 33 A-C. Phase contrast, fluorescence, and dark-field images of an isolated mouse pancreatic acinar cell incubated in TR containing 100 uM CTC. The nuclear area identified in phase contrast is not fluorescent. The position of zymogen granules in the phase contrast and dark-field images (arrows in Fig. 33A and 33C, respectively) correspond to nonfluorescent "shadows" (arrows in Fig. 33B). x 1670.



located granules (arrows) but also provides evidence that incident light did not contribute to the fluorescence image of Fig. 33B. CTC fluorescence appeared in all other areas of the acinar cell including areas of high intensity at the periphery of the cell and in the zymogen granule containing region between granule "shadows." It is not clear which (if any) of these highly fluorescent areas corresponded to the punctate pattern seen in Fig. 32. Such a pattern was not obvious in this cell, possibly because it had faded since the cell had been exposed to incident light for a number of minutes.

11. Fluorescence Microscopy of Acinar Cells During Efflux of Chlorotetracycline

Cells were incubated with CTC in the presence of BSA for 90 minutes, then washed and resuspended in TR* (Ca^{++} = 1.28 mM, Mg^{++} = 0.56 mM) containing no CTC or BSA before microscopy; these conditions were comparable to those under which much of our fluorometry was done. When cells were first placed on a slide they exhibited a uniform yellow green fluorescence with nonfluorescent nuclei and granule-containing regions as described for cells during CTC uptake. Superimposed upon this pattern in a majority of cells were small yellow dots. One could focus up and down through the cell and see that they were distributed throughout its volume. This was particularly apparent where one could focus on spots above and below the nonfluorescent nucleus. This pattern was extremely difficult to photograph because the fluorescence of these spots faded within 15 to 30 seconds of illumination while the uniform pattern, if anything, increased in intensity. In addition cells tended to move during exposure.

Photography became possible after 10 to 15 minutes when cells had adhered to the glass slide. Under these conditions some cells exhibited a brilliant punctate pattern superimposed on a uniform fluorescence of lower intensity. Two cells that showed this pattern

are displayed in Figs. 34A and 34B. Although the relative intensity of the punctate pattern was greater than normal in these cells the majority of cells (in three experiments) exhibited such a pattern. When the cells had become flattened out it became clear that areas of intense fluorescence occurred around the nucleus and at the edge of the granule mass. In Fig. 34C, 3 cells (either undissociated or aggregated after dissociation) containing well-defined nuclei and granular areas (Z) exhibit perinuclear and perigranular areas of intense fluorescence. This distribution is clearly seen in the single cell of Fig. 34D. In this cell distinct, pointlike sources of light occured next to the nucleus and at the periphery of the zymogen granule region, with the highest concentration appearing to be between the nucleus and granule cluster. A few spots occurred within the mass of granules itself. This can also be seen by comparing the fluorescence and phase-contrast images of another cell in Figs. 34E and 34F. In this cell all intense spots were associated with the granule-containing area (whose limits can be seen in the phase-contrast image) or were around the nucleus; again the greatest fluorescence occured at the interface between these two structures.

Although we have not yet been able to identify what structure is responsible for this intense fluorescence, one possibility is that these were mitochondria; this is suggested by the work of DuBuy and Showacre (1961) who observed a pointlike localization of CTC fluorescence at mitochondria in cultured kidney cells and in liver cells. To evaluate this possibility we studied CTC fluorescence from cells containing large numbers of mitochondria: isolated liver cells and rod photoreceptors.

12. Chlorotetracycline Fluorescence From Dissociated Hepatocytes, Erythrocytes, and Photoreceptors

Dissociated hepatocytes showed uniform emission from nonnuclear areas when incubated in 0.1 mM CTC for less than one hour.

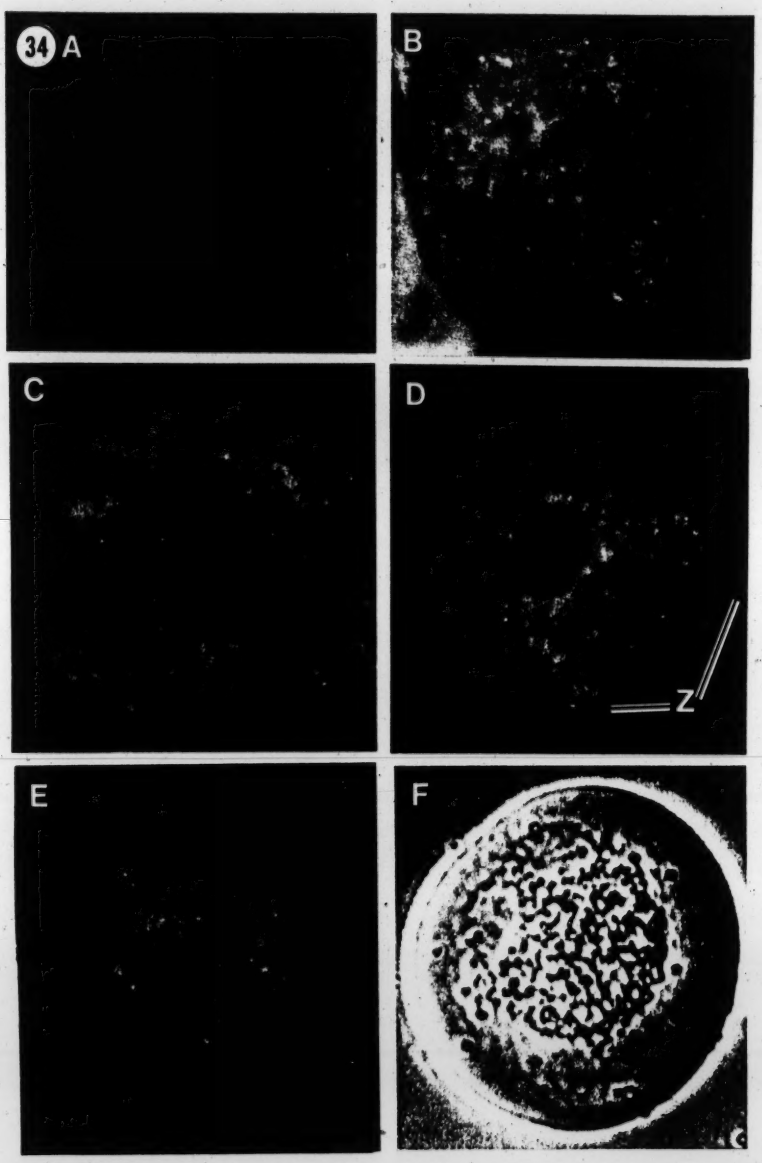
Fig. 34 A-F. Fluorescence of isolated acinar cells during efflux of CTC. Cells were preincubated 90 minutes in TR* containing 100 uM CTC and 1% BSA, then washed and resuspended in TR* containing no CTC or BSA. Micrographs were taken 20 to 40 minutes after resuspension.

A and B. Cells that are still rounded and which display a punctate pattern of fluorescence. x 1440 and x 1480 respectively.

C. Three aggregated cells having an intense fluorescence in the perinuclear areas. Granule clusters are denoted by arrows. x 910.

D and E. Isolated cells displaying punctate fluorescence around the nucleus and zymogen granule areas (Z). x 1520 and x 1470 respectively.

F. Phase contrast image of the cell shown in E. x 1470.


34 A
B
C
D
Z
E
F

Longer incubation produced a distinct punctate pattern of emission as shown in Figs. 35 and 36. In Fig. 36 two types of fluorescence can be distinguished: (a) a uniform emission of low intensity from nonnuclear areas and (b) a punctate emission of high intensity distributed throughout the cell but with lower intensity over the nucleus. Figure 35 shows clearly that the nuclei were nonfluorescent. Upon passing the plane of focus through a hepatocyte, well-focused punctate sources of light filled the cytosol at every plane. Although the punctate pattern varied in intensity from cell to cell, it was in all cases similar to that seen in acinar cell, but much more distinct due to the low intensity of the background fluorescence. Although we did not correlate these points of emission with the position of individual mitochondria, the fact that these isolated hepatocytes were filled with mitochondria (as shown in Fig. 38) suggests this identity.

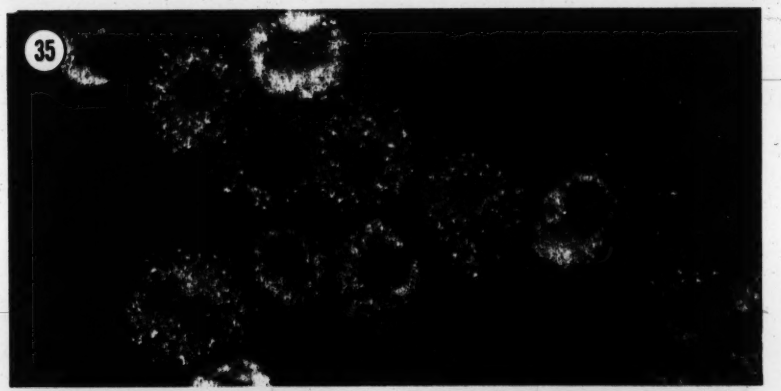
Erythrocytes were used to observe CTC fluorescence from plasma membrane in the absence of mitochondrial or other intracellular membranes. After incubation in 500 uM CTC these cells exhibited a uniform halo of low intensity requiring film exposure 5 to 10 times longer than that used for other cell types (see Fig. 37).

Photoreceptors in the retina offered the opportunity to compare CTC emission from a purely mitochondrial source with that from a purely nonmitochondrial membrane in the same cell. Electron microscopy has shown that the ellipsoid region of the rod inner segment is very tightly packed with mitochondria while the outer segment is exclusively filled with plasma membrane derived disc membranes (Nilsson, 1964). Fig. 39A depicts the CTC fluorescence of the retinal surface from above showing a regular array of photoreceptors. Outer segments are cylindrical and most easily seen at the horizon. The ellipsoid regions of the inner segments (arrows) appear at the base of the outer segments in a regular pattern. Both segments showed intense fluorescence after 30 minutes of incubation in 0.1 mM CTC. The emission from these regions is more easily assessed from Figs. 39B and 39C showing two photoreceptors isolated by crude collagenase digestion of the retina. A phase-contrast image of one

Fig. 35. Fluorescence of isolated rat hepatocytes incubated in TR containing 100 uM CTC. x 950.

Fig. 36. Fluorescence of two isolated rat hepatocytes incubated in TR containing 100 uM CTC. x 1710.

Fig. 37. Fluorescence of human erythrocytes incubated 30 minutes in TR containing 500 uM CTC. x 1600.


35


36

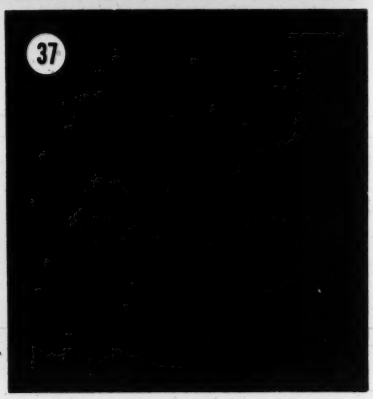

37

Fig. 38. Ultrastructure of an isolated rat hepatocyte suspended in TR. Note the large number of mitochondria in this cell. x 6240.

(38)

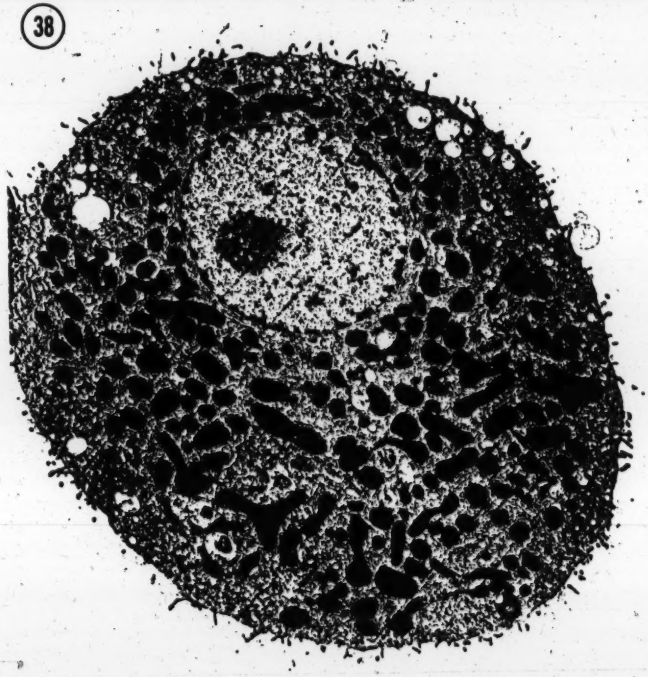


Fig. 39 A-E. Fluorescence and phase contrast images of frog retina and individual photoreceptors isolated from frog retina.

A. Fluorescence of retina incubated in TR (diluted 7 to 9) containing 100 uM CTC. Arrows indicate the ellipsoidal regions of inner segments. x 750.

B and C. Fluorescence of photoreceptors isolated from retina incubated for 30 minutes in OCa TR (diluted 7 to 9) containing 1.3 mg/ml crude collagenase (Worthington) and 100 uM CTC. x 1500.

D. Phase contrast image of the photoreceptor in C. The outer segment (OS), ellipsoidal region (E), and nucleus (N) are labeled. x 1500.

E. Fluorescence of inner segments in frog retina incubated for 60 minutes in TR (diluted 7 to 9) containing 100 uM CTC. x 1600.

39
B
C
D
OS
E
N

(Fig. 39D) identifies the outer segment (OS), ellipsoidal region (E), and nucleus (N). Fluorescence from the outer segment was uniform while that from the ellipsoid region was intense and punctate. This punctate appearance is illustrated clearly in Fig. 39E which shows a group of inner segments (with ellipsoid regions) whose outer segments have been sheared off. This demonstrates that the CTC fluorescence from an identified area rich in mitochondria is intense and punctate.

B. Discussion

This study, by using chlorotetracycline (CTC) as in intracellular probe of divalent cations, provides new, direct evidence for a release of membrane-bound or sequestered calcium during stimulation of pancreatic acinar cells. The utility of CTC as a probe is based on four properties: (a) it enters cells easily and incorporates into cell membranes, (b) its fluorescence in a membrane environment is extremely sensitive to divalent cation chelation, (c) its Ca^{++} and Mg^{++} complexes can be distinguished spectrally, and (d) it can be localized subcellularly by fluorescence microscopy.

Divalent Cation Sensitivity of CTC
Fluorescence from Acinar Cell Membrane

It is already known from model studies with alcohols (Caswell & Hutchison, 1971aa; Caswell, 1972), detergents (Caswell & Hutchison, 1971), and biological membranes (Caswell, 1972; Hallett et al., 1972) that fluorescence of CTC in an amphipathic environment is increased 50 to 200 fold in the presence of divalent cations. In alcohols and micelles of negatively charged detergents CTC forms highly fluorescent complexes with either Ca^{++} or Mg^{++}. However, in biological membranes the fluorescence enhancement is dependent on the relative amounts of membrane-bound Ca^{++} and Mg^{++} as suggested by two observations. First,

micelles of negatively charged detergents that bind divalent cations greatly enhance CTC fluorescence while micelles of neutral detergents which do not bind these ions do not enhance CTC fluorescence (Caswell & Hutchison, 1971a). Second, CTC fluorescence is enhanced by erythrocyte membranes in the presence of calcium but very little in the presence of magnesium (Hallett et al., 1972; Chandler, unpublished observations). This ion specificity must be that of the membrane since CTC fluorescence is enhanced by either Ca^{++} or Mg^{++} in alcohols (Caswell, 1972; Chandler, unpublished observations).

As anticipated, fluorescence of CTC incorporated into membranes of acinar cells was remarkably dependent on divalent cations; addition of calcium to CTC in the presence of sonicated acini rapidly increased fluorescence intensity by at least 60 fold (Fig. 17B). Addition of magnesium resulted in a smaller enhancement possibly reflecting a lower degree of binding of this ion to these membranes. Furthermore, spectral data suggested that fluorescence of CTC in intact pancreatic acini must come from probe molecules complexed to divalent cations. The fluorescence intensity of CTC in acini even in the presence of extracellular EDTA is at least 20 fold greater than that observed for CTC in membranes of acini broken by sonication and exposed to EDTA (Fig. 17A). This implies that CTC fluorescence must be enhanced by divalent cations within the intact cell. This is confirmed by the observation that excitation and emission spectra of cell-associated CTC are similar to those of CTC complexed to calcium in 90% methanol (with maxima at 398 and 529 nm, respectively; see Fig. 18) and unlike those of uncomplexed CTC (maxima at 382 nm and 514 nm in Tris-buffered Ringer's or 382 nm and 534 nm in 90% methanol) or of the divalent cation complexes of CTC in aqueous media (maxima at 382 nm and 520 nm for both complexes). An independent but consistent observation is that the fluorescent ionophore A23187 taken up by isolated acinar cells in the presence of extracellular EDTA exhibited an excitation spectrum like that of the divalent cation complexed molecule (Fig. 5). This suggests that these fluorescent probes easily penetrate the cell and interact with

intracellular Ca^{++} and Mg^{++}, and in the case of CTC this interaction must be in a membrane environment. This conclusion is supported by fluorescence micrographs (Fig. 23 and 33B) showing that structures such as nuclei and the interior of zymogen granules which contain no membranes are nonfluorescent while other areas containing densely packed membranes are fluorescent.

Evidence for Calcium Release During Stimulation

The sensitivity of CTC fluorescence to complexation by the divalent cations present in the membranes of acinar cells makes it reasonable to believe that the loss of CTC fluorescence during stimulation is due to loss of these ions at some intracellular site. This belief is strengthened by the observations that spectra for CTC fluorescence from unstimulated acini are those of CTC complexed to calcium while spectra of stimulated acini are those of CTC complexed to magnesium (see Figs. 20 and 21). This implies that loss of fluorescence during stimulation was due to dissociation of Ca^{++}-CTC complexes probably followed by CTC partitioning out of the membrane site. Participation of the latter mechanism is suggested by the observation that CTC outflux from these cells increased several fold during stimulation (Fig. 24). Both events are consistent with dissociation of Ca^{++}-CTC. Uncomplexed CTC is much less soluble in membranes than is Ca^{++}-CTC and excess CTC formed by dissociation of Ca^{++}-CTC would be expected to leave the membrane. Also, spectra of CTC in methanol (Fig. 18) and in Tris-buffered Ringer's (10 mM, pH 7.4) show that a large intensity loss would be expected to accompany dissociation of Ca^{++}-CTC in an amphipathic environment (Caswell & Hutchison, 1971; Hallett et al., 1972; Chandler, unpublished observations). Studies with isolated mitochondria show that CTC fluorescence from these organelles is also paralleled by CTC content of their membranes (Caswell, 1972). During mitochondrial calcium uptake both CTC fluorescence and content increase and spectra indicate the presence of Ca^{++} complexed CTC. Upon application of an uncoupler,

mitochondrial calcium is released and both CTC fluorescence and content decrease with a parallel time course. At this point, spectra indicate that the CTC remaining in the organelle is complexed to Mg^{++}. This suggests that the amount of calcium sequestered in mitochondria determines how much CTC partitions into the membranes of the organelle, as well as the amount of the calcium-CTC complex that is formed. Thus, the increased exit of CTC from acinar cells during stimulation as well as the decreased size of the CTC pool in stimulated cells (Fig. 25) is consistent with the hypothesis that some set of intracellular membranes release calcium during stimulation and that this release results in dissociation of Ca^{++} complexed CTC (if present already) and a lower affinity of this compartment for CTC. Experiments with ANS and OTC are also consistent with this interpretation. Like CTC, the fluorescence of these probes is enhanced when incorporated into acinar membranes (Fig. 23, and unpublished observations) but unlike CTC neither chelate Ca^{++} or Mg^{++} or exhibit fluorescence that is markedly enhanced by these ions. Acini containing these probes showed no stimulation-induced loss of fluorescence or increased exit of probe so both of these phenomena must stem from the specific ability of CTC to associate with divalent cations in membranes. This rules out the alternative explanation that CTC efflux and fluorescence decrease are simply due to increased permeability of acinar cell membranes to CTC during stimulation. Because amphipathic molecules like CTC cross cell membranes by diffusion rather than specific transport mechanisms (although bacterial membranes may be an exception; see Dockter & Magnuson, 1974) it is quite unlikely that changes in membrane permeability to these molecules would affect CTC but not OTC (an identical molecule with four heterocyclic rings but having an extra hydroxyl group and lacking a chlorine). Furthermore, the first 20 minutes of CTC uptake by acini is not affected by stimulation suggesting that the plasma membrane permeability is not changed and at later times CTC uptake is decreased rather than increased as would be expected if some intracellular compartment were made more permeable to CTC.

Does CTC Monitor a Significant Step in Stimulus-SecretionCoupling?

A number of observations suggest that the calcium release process monitored by CTC is related to stimulus-secretion coupling. First, atropine which is known to block binding of cholinergic agonists to muscarinic receptors blocks both the CTC fluorescence decrease and the amylase release produced by bethanechol, but has no effect on the ability of caerulein to elicit either effect (presumably because it binds to another receptor). Secondly, concentrations of bethanechol which stimulate amylase release also produce fluorescence changes while concentrations too low to stimulate amylase release have no effect on CTC fluorescence.

However, it is also clear from the dose-response relationship that the process monitored is not amylase release itself. Maximal amylase release is achieved at a bethanechol concentration which elicited a fluorescence decrease only 30% of maximal (Fig. 16). In addition, stimulation of amylase release is reduced at supramaximal doses of bethanechol while the fluorescence response remained maximal. These observations argue against the possibility that CTC associates with zymogen granule contents and is simply "secreted" along with the normal products. It is also inconsistent with fluorescence micrographs such as Fig. 33B that show zymogen granule contents are not fluorescent.

It is also unlikely that the depolarization accompanying bethanechol or caerulein stimulation (Nishigama & Petersen, 1975; Poulsen & Williams, 1977b) in itself initiates the process monitored by CTC fluorescence since elevation of extracellular KCl to 58 mM, a concentration known to substantially depolarize acinar cells (Matthews & Petersen, 1973), resulted in no changes in CTC fluorescence. This is an important point because plasma membrane depolarization per se is not sufficient to elicit enzyme release from the exocrine pancreas. If depolarization had produced CTC fluorescence changes, it would have

aroused suspicion that the stimulation-induced process monitored by CTC was peripheral to rather than necessary for triggering of secretion. For CTC fluorescence to directly probe the plasma membrane potential is highly unlikely because this probe, unlike some other fluorescence probes (Waggoner, 1976), is relatively insensitive to membrane potential (Hallett et al., 1972; Cohen, Salzberg, Davila, Ross, Landowne, Waggoner, & Wang, 1974).

Calcium release as monitored by CTC bears some striking similarities to the increased efflux of isotopic calcium seen at the onset of stimulation. Both are initiated with little or no lag (less than 0.5 minutes, which is at least as fast as any rise in amylase can be measured) and both are completed in a relatively short time (it must be noted, however, that the fluorescence response may be terminated by depletion of CTC at the site of release rather than by completion of the calcium release process, a point discussed in more detail below). Dose-response curves show that 3 to 5 fold greater acetylcholine concentrations are required to elicit maximal $^{45}Ca^{++}$ efflux than are required for maximal amylase release. For example, half-maximal amylase release from pancreatic fragments is achieved at about 7×10^{-8} M acetylcholine while half-maximal $^{45}Ca^{++}$ efflux occurs at about 3×10^{-7} M acetylcholine Case & Clausen, 1973; Matthews, Petersen & Williams, 1973). This situation is comparable to that seen in dose-response relationships for bethanechol-induced fluorescence responses and amylase release from pancreatic acini: half-maximal amylase release is obtained with 5 uM bethanechol while half-maximal fluorescence responses require 20 uM bethanechol (see Fig. 16). Thus, increased $^{45}Ca^{++}$ efflux, like CTC fluorescence responses, are seen at doses of cholinergic agonists which elicit enzyme release but are fully developed only at higher doses. These similarities raise the possibility that CTC is probing the calcium release process that is responsible for the sudden increase in $^{45}Ca^{++}$ efflux seen at the onset of secretion. It appears that only a portion of the calcium potentially available for release is required for maximum amylase release.

Comparison of CTC Fluorescence to Other Methods of Detecting Intracellular Calcium Redistribution

The observation that secretagogues rapidly increase $^{45}Ca^{++}$ efflux from slowly exchangable pools and that the release of isotopic calcium is not affected by removal of extracellular calcium (Case & Clausen, 1973; Matthews, Petersen & Williams, 1973; Heisler, 1974; Williams & Chandler, 1975) has been thought to mean that this calcium is bound or sequestered intracellularly. However, localization of this pool by efflux studies has been prevented because efflux is multiphasic and cannot be dissected into 2 or 3 components as has been done for other tissues such as cultured kidney cells and HeLa cells (Borle, 1969a,b, 1970, 1973). This has necessitated the use of cell fractionation and ultrastructural techniques to localize calcium in these cells. Results with these techniques confirm the presence of a number of intracellular calcium pools.

Using cell fractionation, Clemente and Meldolesi (1975a) have shown that the plasma membrane, zymogen granule membranes, smooth-surfaced microsomes, and mitochondria all contain large amounts of calcium while the postmicrosomal supernatant contained very little. (This distribution is in contrast to that for Mg^{++} which is found in rough-surfaced microsomes, mitochondria, and the postmicrosomal supernatant). By fractionating pancreatic fragments which had been loaded with $^{45}Ca^{++}$ and washed for 2 hours in isotope-free media, they found that all of these organelles contained slowly exchangable calcium as indicated by the continued presence of isotope (Clemente & Meldolesi, 1975b). Use of the pyroantimoniate method for ultrastructural localization of calcium (in conjunction with electron probe analysis; Clemente & Meldolesi, 1975a) confirmed some of these observations: the plasma membrane, condensing vacuoles, Golgi sacs, and some mitochondria were heavily labeled with calcium precipitates. In contrast, the endoplasmic reticulum was not labeled, a result in agreement with cell fractionation studies which showed almost no slowly exchangable calcium present in rough-surfaced microsomal

fractions. Although zymogen granules were not labeled in intact tissue, isolated granule membranes were heavily labeled suggesting to the authors that absence of labeling was due to lack of penetration of the precipitating agent rather than a lack of calcium at these membranes.

Both cell fractionation and ultrastructural methods, however, are subject to artifacts. As Meldolesi has stressed (Clemente & Meldolesi, 1975b) there is a substantial amount of calcium redistribution during fractionation, some of which he could correct for. In ultrastructural techniques, fixation and dehydration of the tissue are known to remove diffusible ions unless they are immobilized by precipitation. Thus the reliability of this method depends on precipitation of calcium in a pattern representative of the physiological distribution and maintenance of this pattern during fixation, two conditions that are difficult to achieve or to document.

It is significant then that artifacts inherent in these techniques which "dismantle" the cell can be entirely avoided by using CTC as a probe for membrane-bound divalent cations in living cells. In addition, continuous monitoring of CTC fluorescence intensity and spectral properties allows one to obtain kinetic information like that usually obtained with isotopes, while fluorescence microscopy is able to localize the probe subcellularly, thus providing information usually obtained by cell fractionation or ultrastructural techniques. We took advantage of both properties in experiments aimed at identifying the site of intracellular calcium release occurring during secretory stimulation of pancreatic acinar cells. Before discussing CTC localization of calcium release, however, it is of use to delineate the properties and limitations of this fluorescence probe technique.

One potential problem in the use of CTC is that high doses are known to inhibit mitochondrial respiration (Loomis, 1950; Van Meter, Spector, Oleson & Williams, 1952; Laskin, 1967). However, there are several reasons to believe that CTC did not affect respiration in this and other studies using it as a probe. First, CTC

was used as a probe at concentrations (10 to 100 uM) which are lower than those required to inhibit respiration (200 uM and above; Loomis, 1950; Brody, Hurwitz & Bain, 1954; Laskin, 1967). Secondly, CTC inhibition is seen only in magnesium-depleted mitochondria and can be reversed by addition of magnesium (Van Meter et al., 1952; Brody et al., 1954). Thus, it is unlikely that mitochondria in whole cells would be affected since they are bathed in cytoplasm which is thought to contain about 1 mM magnesium. These arguments are supported by the observation that enzyme secretion by acinar cells--a process known to be almost totally dependent on mitochondrial ATP synthesis (Bauduin et al., 1969; Jamieson & Palade, 1971)-- was essentially unaffected by the presence of CTC (Table 2).

Turning to the spectral properties of CTC, one should first note that CTC fluorescence is enhanced by both calcium and magnesium. This lack of specificity is not a problem in kinetic experiments since the Ca^{++} and Mg^{++} complexes of CTC can be distinguished spectrally as described above. However, this distinction cannot be made by conventional fluorescence microscopy, and the subcellular patterns of CTC emission obtained by this technique could be due to either Ca^{++} or Mg^{++} complexes of CTC.

Second, CTC does not probe all calcium pools in the cell. This stems partly from the fact that CTC selectively monitors membrane-associated calcium, and would not be expected to respond directly to cytoplasmic calcium activities or to Ca^{++} that is bound or precipitated outside of the amphipathic membrane environment. In addition, it is not known whether CTC is present at all sites of membrane-bound calcium or whether it is present at each site in proportion to the amount of ions bound. Most of the experiments in this report were done under conditions of CTC efflux; that is, CTC at sites within the cell was continually flowing into the extracellular space. The amount of CTC present at a given site would depend on how fast CTC washed out which in turn might be determined by its location in the cell, whether it is membrane enclosed, and its divalent cation content. For these reasons, CTC fluorescence changes are probably not

reversible. Once CTC has left the site during calcium release it is no longer present to probe subsequent divalent cation fluctuations at that site. This could account for the observation that atropine can prevent or halt the bethanechol-induced fluorescence decrease but does not reverse it (data not shown). It is also possible that fluorescence changes seen during CTC efflux have a shorter time course than the calcium release process that it monitors. Initial kinetics of the release process are probably monitored faithfully but depletion of CTC at the release site might prevent later stages of calcium release from being visualized.

In contrast, membranes that are in equilibrium with CTC display reversible increases and decreases in CTC fluorescence. A particularly convincing example in isolated mitochondria is shown by Schuster and Olson (1974): 3 successive cycles of aerobiosis-anaerobiosis produce successive, reversible fluctuations in CTC fluorescence. In pancreatic acini under these conditions (after 60 minutes of CTC uptake) the bethanechol-induced fluorescence decrease is still not reversed by atropine in 8 minutes. This suggests that the site depleted of calcium by bethanechol does not rapidly regain these ions, an observation consistent with that of Shelby et al. (1976) that $^{45}Ca^{++}$ tightly bound to acinar cell membranes is rapidly decreased during stimulation but that subsequent addition of atropine allows this calcium pool to be refilled only over a period of 30 minutes.

Despite possible limitations, we chose to use CTC during efflux for the following reasons. First, signal from extracellular CTC is nearly absent. This eliminates signals produced by interaction of ions and drugs with CTC outside the cell which are of no relevance and allows one to obtain spectra from purely cell-associated probe. Secondly, extracellular CTC absorbs incident light thus decreasing the intensity of the cell-associated signal, an unfavorable situation with the amount of noise present in the system. Actually, many of the properties of CTC efflux discussed above are only those normally present when using Ca efflux to detect calcium release. Both CTC

and $^{45}Ca^{++}$ efflux may have the advantage that probe or isotope concentration is reduced at unimportant sites thus accentuating the stimulation-induced changes occurring at a site which loses $^{45}Ca^{++}$ or CTC slowly. CTC, however, has the added advantage that these sites can be located by fluorescence microscopy.

Determination of the Site of Stimulated
Calcium Release by Pharmacologic Agents

Experiments in Fig. 27 indicate that metabolic inhibitors directly or indirectly decrease CTC fluorescence at the same site as does bethanechol. Carbonylcyanide-m-chlorophenylhydrazone (ClCCP), antimycin A, and NaCN all greatly increased the loss of fluorescence in unstimulated cells but had no effect after bethanechol, presumably because the agonist had already triggered calcium and CTC release from the site where these inhibitors usually act to decrease CTC fluorescence (Fig. 27). That all these inhibitors specifically release calcium from mitochondria and block mitochondrial ATP synthesis give strong indication that this site is either mitochondria or is a nonmitochondrial site which binds or sequesters calcium by an ATP-dependent mechanism. For CTC to probe either of these two types of sites is feasible since CTC fluorescence has been shown to monitor calcium sequestration in both isolated mitochondria (Caswell, 1972; Schuster & Olson, 1974; Luthra & Olson, 1976) and in sarcoplasmic reticulum vesicles (Caswell & Pressman, 1972; Caswell & Warren, 1972). Application of FCCP (an uncoupler similar to ClCCP), antimycin A, or cyanide has been shown to release calcium from isolated mitochondria and to rapidly decrease CTC fluorescence from these organelles. Thus, if CTC is present in mitochondria of acinar cells (which is highly likely based on the intense CTC fluorescence from mitochondria in whole cells; see DuBuy & Showacre, 1961; Babcock, First & Lardy, 1976; and Figs 36 and 39) CTC fluorescence of acini should decrease upon application of these inhibitors as was observed. The observation that stimulation blocks the fluorescence changes produced by these

inhibitors suggests that stimulation has a direct effect on mitochondrial calcium stores. The simplest explanation is that stimulation releases calcium from mitochondria since it is difficult to conceive of how calcium release from any other site could prevent an uncoupler or inhibitor of oxidative phosphorylation from releasing mitochondrial calcium.

Experiments with A23187 (Fig. 19) were consistent with the hypothesis that CTC fluorescence monitors divalent cation stores in an intracellular membrane-bound compartment. A23187, in the presence of extracellular calcium, decreases CTC fluorescence even though it is undoubtedly raising cytoplasmic calcium levels under these conditions (which is reflected in increased amylase release, see Figs. 8, 12, and 13). This suggests that A23187 releases calcium from a noncytoplasmic compartment that is monitored by CTC, since membranes exposed to cytoplasm would be expected to bind more calcium rather than release calcium under these conditions. Intracellular release is not unreasonable since A23187, at the low concentrations used in these experiments (1 uM), is known to rapidly deplete calcium and CTC fluorescence of mitochondria in whole cells (Babcock et al., 1976), of isolated mitochondria (Luthra & Olson, 1976) and of sarcoplasmic reticulum vesicles (Caswell & Pressman, 1972). In fact, Lardy and coworkers (Babcock et al., 1976) have demonstrated in sperm that the predominant action of 1 uM A23187 is to rapidly release mitochondrial calcium while higher concentrations (30 uM) produced mitochondrial calcium release followed by net calcium influx. They also showed that CTC fluorescence monitors the mitochondrial calcium release but not the subsequent calcium uptake. This observation supports the conclusion that CTC in acinar cells is monitoring A23187-induced calcium release from an intracellular compartment with the further implication that this could be mitochondria. Thus it is possible that amylase release induced by A23187 under these conditions (see Figs. 8, 12, and 13) is triggered by calcium released from intracellular stores as well as extracellular calcium influx.

In the absence of calcium and magnesium, A23187 addition results in an extremely rapid and complete loss of CTC fluorescence. This may be explained by the fact that A23187 is taken up extremely rapidly under these conditions (see Fig. 3A) and by the possibility that calcium and magnesium may be released from both cytoplasmic and noncytoplasmic compartments. A23187 does induce increased amylase release under these conditions (see Table 2), indicating that ionophore-mediated calcium release from intracellular stores might be able to trigger secretion; however, this conclusion cannot be made with any certainty since A23187 under these conditions may cause cell damage as indicated by release of cytoplasmic enzymes (see Table 2).

A23187, in the absence of extracellular calcium but with magnesium present, does not release divalent cations from CTC probed sites. This is consistant with the observation that the ionophore, even at high concentrations, does not increase amylase release under these conditions (see Fig. 8). Becuase the ionophore complexes magnesium and calcium equally well (Pfeiffer et al., 1974; Pfeiffer & Lardy, 1976) large amounts of magnesium may enter the cell under these conditions. High magnesium levels are known to inhibit the ability of A23187 to release calcium from isolated mitochondria (Binet & Volfin, 1975; Babcock et al., 1976; Pfeiffer, Hutson, Kauffman & lardy, 1976) and from isolated liver cells (Kleineke & Stratman, 1974). Perhaps this accounts for the inability of A23187 to release intracellular divalent cation stores in acinar cells when magnesium but not calcium is present.

Comparison of the fluorescence response in the presence and absence of extracellular calcium and magnesium provided evidence that stimulation-induced fluorescence changes were not secondary to influx of extracellular calcium. Elimination or chelation of these ions extracellularly reduced CTC fluorescence from acini but did not markedly alter the fluorescence response to stimulation with bethanechol or cearulein (see Fig. 2B). It was also observed that the fluorescence decreases produced by mitochondrial inhibitors or A23187 could be elicited in the presence of EDTA. Also, EDTA (unlike

mitochondrial inhibitors) was able to produce a fluorescence decrease either before or after the bethanechol-induced fluorescence decrease (data not shown). These data taken together suggest that the less tightly bound pools of divalent cations that can be rapidly removed by elimination of extracellular calcium and magnesium are separate and distinct from the more tightly bound pool affected by secretagogues and metabolic inhibitors. These data are consistent with the fact that $^{45}Ca^{++}$ released by secretagogues comes from a slowly exchangeable site and is not affected by removal of extracellular calcium (Case & Clausen, 1973; Matthews, Petersen & Williams, 1973; Heisler, 1974; Williams & Chandler, 1975).

From pharmacological studies it appears that there are several pools of divalent cations at least one of which is a membrane-bound compartment. Bethanechol appears to release calcium from the latter which judging from the action of specific mitochondrial inhibitors could be mitochondria or a site requiring ATP synthesized by mitochondria in order to sequester calcium.

Histological Localization of
CTC Fluorescence

Two patterns of CTC fluorescence were observed in acinar cells, a uniform fluorescence which was predominant during uptake of CTC and a punctate pattern seen during CTC efflux. The uniform pattern occurred in two areas of the cell: the extranuclear spaces outside of the granule cluster, and between individual granules within the zymogen granule cluster (see Fig. 33A-C). Electron micrographs show that the areas in acinar cells that are outside of the granule cluster are filled with rough endoplasmic reticulum while the zymogen granule cluster is interlaced with Golgi sacs and small, smooth-surfaced vesicles (see Fig. 30). In contrast, the punctate pattern (Fig. 34) suggests intense CTC emission from small, individual organelles such as mitochondria or Golgi vesicles. The pattern was seen either scattered throughout the cell (Figs. 34A and 34B) or in

cells somewhat spread out was clearly associated with the perinuclear and perigranular areas (Fig. 34D and 3E). Electron micrographs (e.g. Fig. 30) show that mitochondria in isolated acinar cells are distributed throughout the cell but are found in particularly great frequency in the perinuclear and zymogen granule containing areas. Likewise, golgi membranes are almost entirely associated with the granule cluster or lie between the nucleus and granule cluster. While precise morphometric techniques would be required to make any definitive correlations, these observations do suggest that distribution of either mitochondria or the small vesicles of the Golgi regions is consistent with these being a source of punctate CTC fluorescence.

Patterns of CTC fluorescence from other cell types strengthen these correlations. The outer segments of photoreceptors contain disc membranes packed densely as are rough endoplasmic reticulum membranes in acinar cells and exhibit a uniform fluorescence (Fig. 39) similar to that seen in nongranular areas of acinar cells. This uniform pattern is particularly well demonstrated in the acinar cell of Fig. 34D in which the uniform fluorescence encircling the nucleus and granule area is distinct both in appearance and space from the punctate pattern in the perinuclear and perigranular areas. On the other hand, the punctate pattern is similar to the point-like CTC emission from the ellipsoidal region of photoreceptors and from the cytoplasm of hepatocytes (see Fig. 36 and 39). That the distribution of point-like emission is identical to that for mitochondria in these cells and is not seen in erythrocytes which have no mitochondria (Fig. 37) suggest mitochondria are one possible source of this pattern in acinar cells. These data confirm and extend the observations of DuBuy and Showacre (1961) showing that point-like CTC fluorescence from cultured kidneys was specifically localized in mitochondria that had been individually identified by phase contrast microscopy.

Possible Origins of the Calcium Released During Stimulation as Determined by CTC Fluorescence and Other Methods

Pharmacological and histological techniques have both provided evidence that the secretagogue-induced release of calcium monitored by CTC could come from mitochondria. There is in fact other evidence that mitochondrial calcium storing ability may be altered during stimulation. Clemente and Meldolesi (1975b) have studied the $^{45}Ca^{++}$ distribution in subcellular fractions obtained from pancreas which had been incubated in isotope for 60 minutes than superfused in isotope-free medium for 150 minutes to remove rapidly exchanging isotope. Under these conditions (as discussed above) most of the tracer appears in the mitochondrial, smooth-surfaced microsomal, plasma membrane, and zymogen granule membrane fractions. Presence of caerulein during the last 30 minutes of incubation decreased the isotope content of the mitochondrial fraction by 50% while the $^{45}Ca^{++}$ contained in other fractions was unchanged. This reduction occurred rapidly since almost identical results were obtained after 5 minutes of stimulation. This time course (as far as one can conclude from the data available) is compatible with the requirement that the CTC-monitored release process be observable within the first few minutes of stimulation.

Mitochondria isolated from pancreas, like those from other tissues, are known to have efficient calcium-accumulating mechanisms (Carafoli & Lehninger, 1971; Argent, Smith & Case, 1976). Electron micrographs of mitochondria in pancreatic fragments display numerous dense granules in their matrix (Ichikawa, 1965; Chandler, unpublished observations) which from studies in isolated mitochondria (Greenawalt, Rossi & Lehninger, 1964; Sutfin, Holtrop & Ogilvie, 1971) are thought to represent deposits of divalent cations. This possibility is supported by the observation that the number, size, and density of the granules increase when pancreatic fragments are incubated in Sr^{++} or Ba^{++}, divalent cations that may bind at calcium binding sites but

which are more electron dense (Chandler, unpublished observations). It is of interest to note that Sr^{++} and Ba^{++} deposits were formed only in mitochondria and (less frequently) in lysosomes, with no evidence for binding at other intracellular sites. In addition, at least one investigator has claimed that there is a reduction in number and size of mitochondrial granules during cholinergic stimulation of enzyme release (Ichikawa, 1965).

A possible alternative to mitochondria as a site of secretagogue-induced calcium release is the Golgi apparatus or other smooth-surfaced vesicles found in the zymogen granule-containing region. Evidence for this is largely histological; that is, intense CTC fluorescence comes from membranes between individual zymogen granules and between the granule cluster and nucleus, regions known from electron micrographs to contain smooth-surfaced membranes. Subcellular fractions of smooth microsomes isolated from some secretory cells are known to have ATP-dependent calcium accumulating abilities and similar fractions from pancreas do contain large amounts of slowly exchangable calcium (Clemente & Meldolesi, 1975b). However, it has not been possible to demonstrate energy-dependent calcium transport in the fractions from pancreas (Argent, Smith & Case, 1975) or that the slowly exchangable $^{45}Ca^{++}$ content of this fraction is altered by stimulation (Clemente & Meldolesi, 1975b).

Although plasma and zymogen granule membranes appear to have large amounts of calcium associated with them (Clemente & Meldolesi, 1975a) there is presently no clear evidence on whether this calcium is important for stimulus-secretion coupling. It is unlikely that calcium release from either of these membranes could account for the stimulation-induced changes in CTC fluorescence. Although it was possible to detect CTC fluorescence from the etythrocyte plasma membrane (Fig. 37), this membrane probably contributes very little to the fluorescence of the acinar cell and could not account for the 30% loss of intensity that secretagogues trigger. Also, the CTC pool altered by secretagogues is relatively unaffected by extracellular divalent cation levels--an unlikely characteristic of CTC at the

plasma membrane. CTC fluorescence in granule-containing regions is patchy or absent (Fig. 34D and 34E) which is quite unlike the dense packing of granules seen in phase contrast images (Figs. 33A and 34F). This suggests that CTC does not associate with granule contents or membranes to any large extent. The possibility that calcium or CTC bound to zymogen granule membranes is released during the exocytotic event (for example when the granule membrane is incorporated into the plasma membrane) is completely untenable since stimulation of CTC fluorescence loss and $^{45}Ca^{++}$ efflux have a different dose-response relationship than does amylase release. Furthermore, stimulation in the presence of vinblastine produces a normal increase in $^{45}Ca^{++}$ efflux (Williams & Lee, 1976) but no amylase release while application of A23187 to an isolated rabbit pancreas produces amylase release with almost no increased $^{45}Ca^{++}$ efflux (Schreurs, et al., 1976a).

Nor is it known if rough endoplasmic reticulum membranes are a source of trigger calcium. Areas of the acinar cell containing rough endoplasmic reticulum have quite significant CTC fluorescence and therefore it is possible that alterations in the fluorescence of these membranes could produce changes in the total cell intensity. However, it seems unlikely that this is the source of the stimulation-induced fluorescence changes since Clemente and Meldolesi (1975a) have shown that rough microsomal fractions of pancreas contain exceedingly small amounts of slowly exchangable calcium (as indicated by $^{45}Ca^{++}$ retention) and that this small amount is not affected by stimulation (Clemente & Meldolesi, 1975b). These membranes do contain large amounts of magnesium (Clemente & Meldolesi, 1975a) and although this appears to be associated with the ribosomes rather than the membrane itself, some CTC fluorescence from these membranes may be that of the magnesium-CTC complex rather than the calcium complex of CTC.

Evaluation of Mitochondria as a Site of Secretagogue-Induced Calcium Release and CTC Fluorescence Changes

Because histological and pharmacological data have suggested that mitochondria may be the origin of stimulation-dependent fluorescence changes, it is useful to review the characteristics of calcium sequestering by these organelles and how CTC might interact with them.

An "energized" state of mitochondria, created by substrate oxidation and subsequent electron transport via the respiratory chain of carriers, can be used to transport calcium, magnesium, or manganese ions into mitochondria as an alternative to use of the energy to synthesize ATP (Lehninger, 1970; Carafoli, 1973). One hundred to 150 nanomoles of calcium per mg mitochondrial protein are taken up by an energy-linked process in the absence of permeant anions (Carafoli, 1973). Uptake is rapid, having a half-time of about 15 seconds (Mela & Chance, 1968) and is coincident with binding of calcium to a small number of high affinity sites (Reynafarje & Lehninger, 1969) that are thought to constitute part of an electrogenic uniport system for transport of calcium into mitochondria (Lehninger, 1974; Puskin, Gunter, Gunter & Russell, 1976). This identity is based on the observation that both binding and transport have a similar dissociation constant (K_d and K_m are 1 to 5 uM), have the same specificity for divalent cations (calcium, magnesium, and manganese), are both inhibited by low concentrations of lanthanum and ruthenium red, and are both absent from mitochondria of certain species (Carafoli, 1973).

There is now general agreement that the "energized" state of mitochondria consists of an alkaline-inside proton gradient (Mitchell & Moyle, 1967; Mitchell, 1968; Skulachev, 1971) and an inside-negative membrane potential of -150 to -200 mV (as determined, for example, by using valinomycin to measure the potassium gradient (Rottenberg, 1975)). In this case, mitochondria could accumulate large amounts of

calcium if the ion was distributed according to the electrical gradient.

Calcium uptake is facilitated by permeant anions such as phosphate, acetate, or bicarbonate (Mela & Chance, 1965; Luthra & Olson, 1976). Lehninger (1974) found that anions must be proton-donating in order to have this effect and suggested that they play a role (yet to be defined) in transducing energy contained in the proton gradient into a form used to power calcium uptake. In addition, phosphate coprecipitates with calcium that has been transported into the matrix to form calcium phosphate deposits that can be detected either ultrastructurally as "mitochondrial granules" or by an increase in mitochondrial density (Greenawalt, Rossi & Lehninger, 1964). Formation of these deposits is thought to allow large amounts of calcium to be accumulated without a rise in the calcium activity of the matrix.

Calcium efflux from mitochondria is also rapid. Application of uncouplers, inhibitors of electron transport, or inhibitors of calcium uptake (such as lanthanum or ruthenium red) result in loss of isotopic calcium from isolated mitochondria within 3 to 5 minutes (Luthra & Olson; Puskin et al., 1976) and rapid loss of calcium accumulated by whole cells such as sperm (Babcock et al., 1976) and Erhlich acites tumor cells (Cittadini, Scarpa & Chance, 1971). Rapid efflux is compatable with the idea that the large calcium gradient across the mitochondrial membrene is maintained by the membrane potential. It is inconsistant with the early model of Chance (1965) in which calcium is pumped against a concentration gradient maintained by a low membrane permeability. This would require that the rate constant for calcium efflux be several orders of magnitude lower than that for uptake. This appears not to be the case. In fact, Puskin et al. (1976) have proposed the opposite: there may be a mechanism that transports calcium <u>out</u> of mitochondria to somewhat reduce the calcium gradient created by the membrane potential.

It is possible then that mitochondria could rapidly release calcium to produce a transient rise in cytoplasmic calcium activity

which would serve as a mechanism for stimulus-secretion coupling. If the mitochondrial calcium gradient is maintained by the membrane potential, release of calcium could be triggered either by inhibition of calcium uptake, stimulation of calcium transport out of mitochondria, or by lowering the mitochonrial membrane potential. The amount of calcium contained within mitochondria would be easily sufficient to do the job if one considers the following: a) cytoplasmic calcium activity is $\leq 10^{-6}$ M; b) intramitochondrial calcium activity is about 1 mM (based on the solubility of calcium phosphates like those in mitochondrial granules (Mac Gregor & Nordin, 1960)); and c) the ratio of cytosol volume to mitochondrial volume in pancreas is 7:1 (Bolender, 1974). Complete release of calcium stored in mitochondria (not counting that in mitochondrial granules) would raise the cytoplasmic calcium activity to above 10^{-4} M if calcium were not bound or transported out of the cell too rapidly.

To understand whether the data presented in this study are consistant with a release of mitochondrial calcium it is necessary to consider the mechanism by which CTC might probe calcium transport in these organelles. From model studies with erythrocyte membranes, detergents, and alcohols it is clear that CTC fluorescence is not enhanced by hydrophobic environments unless divalent cations are present (Caswell & Hutchison, 1971a; Hallett et al., 1972). This plus spectral evidence that CTC fluorescence from pancreatic acinar cells is that of its calcium complex in a membrane environment (Fig. 18) make it reasonable to believe that CTC monitors calcium binding to mitochondrial membranes in acinar cells. This is supported by studies using CTC in isolated mitochondria. If low doses of A23187 are applied to mitochondria, magnesium is lost initially while calcium is retained until the oxygen supply is exhausted (Binet & Volfin, 1975; Pfeiffer, Hutson, Kauffman & Lardy, 1976). In this case, CTC fluorescence changes parallel the loss of calcium but not that of magnesium. In addition, ruthenium red which selectively inhibits calcium uptake (probably by displacing calcium bound to membrane transport sites (Carafoli, 1973)) rapidly decreases CTC fluorescence

from mitochondria (Pfeiffer et al., 1976). However, it is unlikely that CTC can monitor calcium once it has left the mitochondrial membrane and entered the matrix or precipitated as a phosphate in mitochondrial granules. In fact, Luthra and Olson (1976) have shown that addition of phosphate to isolated mitochondria (which probably coprecipitates with calcium in the matrix) increases calcium uptake but decreases CTC fluorescence.

As an alternative, one could ask whether CTC monitors other aspects of mitochondrial function such as respiration, the pH gradient, or the membrane potential, all of which could undergo changes upon alteration of calcium transport. A number of fluorescence probes are known to monitor the energized state of mitochondria. The fluorescence of ANS, for example, is thought to respond to mitochondrial energization because a membrane conformational change alters ANS binding sites by increasing their hydrophobicity and their affinity for the probe (Azzi, Chance, Radda & Lee, 1969; Radda, 1971; Azzi & Santato, 1972; Ballard, Barker, Barrett Bee, Dwek, Radda, Smith & Taylor, 1972). A second type of probe, as exemplified by the lipophilic cation, ethidium (Azzi & Santato, 1972), and the cyanine dyes (Laris, Bahr & Chaffe, 1975) are considered to respond directly to changes in the mitochondrial membrane potential (Skulachev, 1971; Azzi & Santato, 1972; Waggoner, 1976).

A number of observations make it unlikely that CTC is reacting to the mitochondrial energy state as are these probes. Addition of succinate to mitochondria in the absence of calcium induces an energization as indicated by ANS fluorescence (Azzi et al., 1969), and an increase in membrane potential as indicated by a cyanine dye (Laris et al., 1975), but produces no change in CTC fluorescence (Schuster & Olson, 1974; Luthra & Olson, 1976). Conversely, depletion of calcium and magnesium from isolated mitochondria by high doses of A23187 is accompanied by a rapid decrease of CTC fluorescence but ANS fluorescence is not affected (Schuster & Olson, 1974). Also, Pfeiffer et al. (1976) have shown that low doses of A23187 stimulate mitochondrial respiration (presumably due to continual reuptake of

lost calcium) but net calcium loss and CTC fluorescence changes are produced only by higher doses of A23187. Thus CTC does not respond in a manner similar to fluorescence probes commonly used to detect changes in membrane potential or energy state.

It is more difficult to evaluate the effects of the mitochondrial pH gradient on CTC fluorescence because no studies have measured both CTC fluorescence and intramitochondrial pH. Although the fluorescence of the magnesium complex of CTC is independent of pH in the range of 7 to 9, its association constant increases with pH (Caswell, 1972). This is logical if one considers the following reaction:

$$CTC + Mg^{++} \rightleftharpoons CTC\text{-}Mg^{++} + nH^{+} \quad (n \sim 2 \text{ at pH } 7.4)$$

This means that alkalinization of mitochondria during energization or calcium uptake would promote the formation of CTC-divalent cation complexes while acidification of energized mitochondria by permeant anions (Rasmussen, Chance & Ogata, 1969) would favor dissociation of CTC complexes. However, there is evidence that divalent cation uptake and release rather than changes in the pH gradient are the major determinent of CTC fluorescence changes: responses of CTC fluorescence to substrate or inhibitor addition are considerably attenuated in the absence of extramitochonrial calcium (Schuster & Olson, 1974; Luthra & Olson, 1976) or when calcium uptake is inhibited by a lanthanide (Caswell, 1972).

I prefer to believe, then, that the stimulation-induced decrease of CTC fluorescence from pancreatic acinar cells results from a decrease in calcium binding to mitochondrial membranes. The simplest explanation is that mitochondrial calcium is released (the possibility that increased uptake of phosphate could "pull" calcium from these membranes into mitochondrial granules can not be ruled out; however, it is difficult to see how this mechanism would produce the transient release of isotopic calcium seen during pancreatic stimulation). Calcium release could be secondary to deenergization of mitochondria but this is unrealistic in view of the fact that pancreatic secretagogues increase respiration (Dickman & Morrill,

1957) while ATP levels remain essentially normal (Bauduin et al., 1969; Williams, 1977). However, one can not eliminate the possibility that an increased demand on ATP synthesis lowers the steady state energy level in mitochondria and thereby releases calcium. This could be tested by monitoring the redox state of respiratory chain components by spectroscopy since a greater energy demand should oxidize these carriers. In fact, NADH oxidation has been observed in a number of tissues in response to hormones and has been interpreted in this manner (Chance, Schoener & Ferguson, 1962; Williamson & Jamieson, 1965).

A more exciting explanation is that secretagogues or hormones (via intracellular messengers) can release mitochondrial calcium by specifically inhibiting calcium uptake or facilitating calcium efflux. In fact, there is already evidence that parathyroid hormone is able to increase efflux of calcium from mitochondria in kidney cells (Borle, 1972). Application of parathyroid hormone to kidney cells raises cyclic AMP levels and increases calcium efflux from a slowly exchangable pool that appears to be mitochondria (Borle, 1971, 1973). The effects on calcium efflux in whole cells can be mimicked by application of exogenous cyclic AMP (Borle, 1973) and if this nucleotide is applied to mitochondria isolated from kidney cells, it stimulates a net calcium efflux which raises the medium calcium concentration from 10^{-5} M to 10^{-4} M within a few seconds (Borle, 1974). In this context it is intriging to note that the transient release of isotopic calcium seen when acetylcholine or CCK-PZ is applied to pancreatic acinar cells closely resembles that seen when cAMP is applied to kidney cells (Borle, 1973). Perhaps pancreatic secreatagogues, like parathyroid hormone, are able to facilitate calcium efflux from mitochondria and thereby increase the cytosol calcium activity. While more study is needed to put this hypothesis on firm ground, it is in complete agreement with the secretagogue-induced effects on CTC fluorescence observed in this study.

V. CONCLUSION

This study has used the divalent cation ionophore A23187 and the antibiotic chlorotetracycline as fluorescence probes to study calcium redistribution during stimulus-secretion coupling in living pancreatic exocrine cells. By preparing a standard Aminco-Bowman spectrofluorometer so that dissociated cells or acini could be stirred and kept at 37° in a cuvette, it was possible to obtain a continuous recording of the fluorescence of these probes while incorporated in the membranes of these cells. This study is the first to directly measure the time course and distribution of A23187 uptake in whole cells. Fluorometric studies showed that uptake of ionophore was extremely rapid in the absence of divalent cations but was markedly retarded in the presence of calcium or magnesium. A23187 taken up by acinar cells was largely incorporated into intracellular membranes as judged by fluorescence microscopy and interacted with divalent cations within the cell as judged by its excitation spectrum.

The ionophoric properties of A23187 were used to test the possibilities that A23187 could trigger secretion by a) increasing the plasma membrane permeability to calcium thereby producing an increased calcium influx, and b) releasing intracellular stores of calcium. The fact that A23187-mediated amylase release (in the presence of magnesium) is dependent on extracellular calcium suggested that the ionophore does promote an increased influx of calcium which triggers secretion. It was also clear from experiments using CTC that A23187 can deplete intracellular stores of divalent cations and that it can increase amylase release even in the absence of extracellular calcium and magnesium. However, the ability of A23187 to cause cytolysis prevented any conclusions as to whether intracellular calcium release by the ionophore was responsible for triggering amylase release.

Chlorotetracycline was used as a non-perturbing probe for divalent cations in pancreatic acini to obtain new evidence for intracellular calcium redistribution during secretory stimulation. Both pharmacological and histological evidence has been presented

which suggests or is consistent with the hypothesis that calcium is released from mitochondria or possibly another ATP-dependent, calcium-sequestering organelle. The major pieces of evidence supporting this hypothesis are the following:

1. CTC fluorescence in pancreatic acini is rapidly decreased by bethanechol or caerulein through a receptor-mediated event that bears the same dose-response relationship to amylase release as does stimulation-induced release of $^{45}Ca^{++}$. These events occur in the presence or absence of extracellular calcium.

2. Spectral data indicate that there is a preferential loss of calcium-complexed CTC during this process.

3. CTC is known to localize specifically in mitochondria of whole cells and to display fluorescence which is proportional to the calcium sequestered by these organelles.

4. Metabolic inhibitors known to release calcium and decrease CTC fluorescence of mitochondria appeared to decrease CTC fluorescence of acinar cells at the same site as does bethanechol.

5. Fluorescence microscopy of isolated acinar cells during CTC efflux indicates presence of an intense punctate emission similar to that seen from mitochondria in other cell types.

While evidence was presented that the calcium release process studied does not require extracellular calcium, it should be emphasized that none of the data limit or delineate the role of extracellular calcium in stimulus-secretion coupling. There is growing evidence that extracellular calcium either modulates (Chandler & Williams, 1974; Williams & Chandler, 1975) enzyme release or by an increased calcium influx (Kanno & Nishimura, 1976; Kondo & Schulz, 1976a,b) acts in concert with intracellular calcium release to trigger secretion.

Thus, data in this study substantiate that intracellular calcium release occurs during stimulus-secretion coupling but give no reason to believe that this is the only way inwhich calcium is involved.

The present study, to our knowledge, is the first to use CTC as a probe for calcium redistribution during stimulus-secretion coupling but it is clear already that this probe could be of use in other secretory systems. Although this technique is most easily carried out with isolated or dissociated cells, it may in the future be extended to intact tissue by use of reflectance fluorometry. CTC fluorescence allows continuous monitor of membrane associated Ca^{++} and Mg^{++} during secretion as well as pharmacological and histological localization of divalent cation stores in the living cell.

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